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The Role of the Endocannabinoid System in the Emotional Modulation of Pain

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Abstract

Fear-conditioned analgesia (FCA) is the robust suppression of pain that occurs upon re-exposure to a context previously paired with an aversive stimulus. FCA is mediated by activation of the descending inhibitory pain pathway. The medial prefrontal cortex (mPFC) in rodents is typically subdivided into three subregions; the infralimbic (IL), prelimbic (PrL) or anterior cingulate (ACC) cortices. While evidence exists of a role for the endocannabinoid system in the mPFC in FCA, further investigation is warranted. Moreover, few studies have investigated the role of genetic background on the emotional modulation of pain. The Wistar-Kyoto (WKY) rat strain represents a useful model to investigate the role of genetic background on FCA. This rat strain displays an anxiodepressive-like phenotype and is highly sensitive to both stress and pain. The overall aims of the work described in this thesis were (1) to further examine the role of the endocannabinoid system in the three subregions of the mPFC in FCA and (2) to compare FCA, conditioned fear and pain-related behaviour in WKY versus Sprague-Dawley (SD) rats and investigate the role of the endocannabinoid system in the mPFC in the differential behavioural profile of these two strains.

FCA was modelled by combining the formalin test of persistent pain with classical Pavlovian fear conditioning to context in rats. Male Lister-Hooded rats were used for the initial studies. On conditioning days, rats received footshocks (10x1s, 0.4mA) spaced 1 minute apart in a conditioning arena. Control rats received no footshocks and were placed in the conditioning arena for an equivalent amount of time (10-minutes). 23.5 hours later, rats received an intraplantar injection of formalin (2.5%, 50 μ l) into the right hind paw. 30-minutes later rats were re-exposed to the contextually aversive footshock arena and behaviours were recorded for 30-minutes. In some of these experiments, rats had guide cannulae implanted into the left and right IL, PrL and ACC approximately one week before testing. On the day of testing, 15 minutes prior to re-exposure to the arena, depending on the experiment, rats received bilateral intracerebral microinjections of the fatty acid amide hydrolase (FAAH) inhibitor, URB597, or the CB₁ receptor antagonist, AM251, or the monoacylglycerol lipase inhibitor (MAGL), MJN110, or the CB₂ receptor antagonist, AM630, or a combination of AM251+URB597 or MJN110+AM251 or MJN110+AM630. Additional experiments compared FCA in male WKY and SD rats and examined the role of AEA and CB₁ in the IL of WKY and SD rats in response to inflammatory pain. Rats were implanted with cannulae into the left and right IL approximately one week before testing. Formalin-evoked nociceptive behaviour was measured

for 1 hour following bilateral intra-IL microinjection of the AEA analog methanandamide, m-AEA, or URB597 or AM251 or the CB₁ agonist ACEA.

In the ACC, neither FAAH inhibition nor CB₁ antagonism had any effect on the expression of FCA or contextually induced fear. Intra-IL, PrL or ACC administration of the MAGL inhibitor MJN110 significantly attenuated the expression of FCA, effects unopposed by co-administration of the CB₁ antagonist, AM251. MJN110 elevated levels of 2-AG in the ACC but not in the IL or PrL. The attenuation of FCA by intra-ACC MJN110 was blocked by co-administration with the CB₂ antagonist, AM630. Alone, AM630 significantly reduced formalin-evoked nociceptive behaviour in non-fear-conditioned but not fear-conditioned rats. These data provide new evidence to support a role for MAGL substrates in the IL, PrL ACC, in the modulation of FCA. The data suggest that FCA is attenuated by 2-AG-CB₂ receptor signalling in the ACC, and that CB₂ receptors in the ACC may facilitate formalin-evoked nociceptive behaviour.

WKY rats exhibited reduced expression of FCA and increased formalin-evoked nociceptive behaviour compared to SD rats; suggesting an impairment in the descending inhibitory pain pathway of this strain. WKY rats also had lower levels of AEA in the IL cortex, compared with SD rats. In SD rats, intra-IL administration of m-AEA and URB597 reduced formalin-evoked nociceptive behaviour an effect not seen in WKY rats. Intra-IL administration of AM251 and ACEA had no effect on formalin-evoked nociceptive behaviour in either SD or WKY rats. These results indicate differences in the endocannabinoid system in the IL of WKY rats compared to SD controls, which may account for the hyperalgesic phenotype exhibited by this strain.

In conclusion, the data provide further evidence for an important physiological role of the endocannabinoid system within the mPFC in FCA, and formalin-evoked nociceptive behaviour. This work enhances our understanding of the mechanisms underlying emotional modulation of pain and may facilitate the development of new therapeutic strategies for pain- and fear-related disorders and their co-morbidity.

Author's Declaration

I hereby declare that the work presented in this thesis was carried out in accordance with the regulations of the National University of Ireland Galway. The research is original and entirely my own with the following exceptions:

1. Initial surgeries for chapter 2 were performed by Dr. Kieran Rea for training purposes
2. Conditioning data for chapter 6 was rated by Ms. Aoife Campbell

The thesis or any part thereof has not been submitted to any other institution in connection with any other academic award. Any views expressed herein are those of the author.

Signed: _____

Date: _____

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Abbreviations

2-AG: 2-arachidonoyl glycerol

5HT: Serotonin

ABA: Accessory basal nucleus of the amygdala

AC: Adenylate cyclase

ACC: Anterior cingulate cortex

AEA: Arachidonyl ethanolamide / Anandamide

ANOVA: Analysis of variance

ATP: Adenosine triphosphate

BDNF: Brain derived neurotropic factor

BLA: basolateral amygdala

cAMP: Cyclic adenosine monophosphate

CB1: Cannabinoid type 1 receptor

CB2: Cannabinoid type 2 receptor

CCI: Chronic constriction injury

CeA: Central nucleus of the amygdala

CFA: Complete Freund's adjuvant

CNS: Central nervous system

COMT: Catechol-O-methyltransferase

CPS: Composite pain score

CS: Conditioned stimulus

CUS: Chronic unpredictable stress

DAG: 1,2-diacylglycerol

DAGL: Diacylglycerol lipase

DHSC: Dorsal horn of the spinal cord

dIPAG: Dorsolateral periaqueductal grey

DMSO: dimethyl sulfoxide

dmPAG: Dorsomedial periaqueductal grey

dPAG: Dorsal periaqueductal grey

DRG: Dorsal root ganglia

FAAH: Fatty acid amide hydrolase

FABPs: Fatty acid binding proteins

FC: Fear-conditioned

FCA: Fear-conditioned analgesia

FLAT: FAAH-like anandamide transporter

fMRI: Functional magnetic resonance imaging

GABA: Gamma-Aminobutyric acid

GiA: Gigantocellular reticular nucleus

GPCRs: G-protein coupled receptors

GPR119: G protein-coupled receptor 119

GPR55: G protein-coupled receptor 55

HMBA: 4-hydroxy-3-methoxybenzylamine

HPA: Hypothalamic–pituitary–adrenal

i.c.v.: Intracerebroventricular

IL: Infralimbic

i.pl: Intra-plantar

ITC: Intercalated cell mass

JNK1: c-Jun N-terminal kinase 1

JNK2: c-Jun N-terminal kinase 2

KO: Knockout

LA: Lateral nucleus of the amygdala

LC-MS/MS: liquid chromatography coupled to tandem mass spectrometry

IPAG: Lateral periaqueductal gray

lyso-PLC: lyso-phospholipase C

M2: Motor cortex

MAGL: Monoacylglycerol lipase

MAPK: Mitogen-activated protein kinase

MeA: Medial nucleus of the amygdala

mGluR: Metabotropic glutamate receptor

mPFC: medial prefrontal cortex

NADA: N-arachidonoyl-dopamine

NAPE: N-acylphosphatidylethanolamine

NAPE-PLD: N-acyl phosphatidylethanolamine-specific phospholipase D

NAT: N-acyltransferase

NFC: Non-fear-conditioned

NIH: National Institute of Health

Noladin ether: 2-arachidonoyl glycerol ether

NSAIDS: Non-steroidal anti-inflammatory drugs

OEA: N-oleoylethanolamide

OX: Orexin

PAG: Periaqueductal grey

PBN: Parabrachial nucleus

PC: Phosphatidyl choline

PCR: Polymerase chain reaction

PE: Phosphatidylethanolamine

PEA: N-palmitoyl-ethanolamide

PET: Positron emission tomography

PFC: Prefrontal cortex

PI: Phosphatidylinositol

PKA: Protein kinase A

PLA1: Phospholipase A1

PLC: Phospholipase C

PPARs: Peroxisome proliferator-activated receptors

PrL: Prelimbic

PTSD: Post-traumatic stress disorder

QTL: Quantitative trait loci

RVM: Rostral ventromedial medulla

SD: Sprague-Dawley

SIA: Stress-induced analgesia

SIH: Stress-induced hyperalgesia

SNI: Spared nerve injury

SNL: Spinal nerve ligation

SNPs: Single nucleotide polymorphisms

THC: Δ 9-tetrahydrocannabinol

TRPV1: Transient receptor potential vanilloid 1

US: Unconditioned stimulus

VEH: Vehicle

VH: Ventral hippocampus

Virodhamine: O-arachidonoyl ethanolamine

vlPAG: Ventrolateral periaqueductal gray

VPL: Ventroposterolateral thalamus

WKY: Wistar-Kyoto

List of publications and conference proceedings

Publications

Original research papers (peer reviewed):

Rea K, McGowen F+, **Corcoran L**+, Roche M, Finn DP. The prefrontal cortical endocannabinoid system modulates fear-pain interactions in a subregion-specific manner. *British Journal of Pharmacology*. 2018 May 30. doi: 10.1111/bph.14376. + Joint second authorship.

Henry RJ, Kerr DM, Flannery LE, Killilea M, Hughes EM, **Corcoran L**, Finn DP, Roche M (2017) Pharmacological inhibition of FAAH modulates TLR-induced neuroinflammation, but not sickness behaviour: An effect partially mediated by central TRPV1. *Brain Behav Immun*. 2017 May;62:318-331

Original research papers in preparation :

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Book Chapter

Corcoran. L, Roche M, Finn DP (2015). The Role of the Brain's Endocannabinoid System in Pain and Its Modulation by Stress. In: *International Review of Neurobiology, Endocannabinoids*, Volume 125, p203-55. Editors: Loren Parsons and Matthew Hill © 2015 Elsevier Inc. doi:10.1016/bs.irn.2015.10.003

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- 1) **Corcoran. L**, Roche M, Finn DP (2018). Regulation of nociceptive behaviour and fear-conditioned analgesia by 2-AG in the rat medial prefrontal cortex. 28th Annual Symposium of the International Cannabinoid Research Society. Oral Presentation
- 2) **Corcoran. L**, Rea. K, Roche M, Finn DP (2017). Hyperalgesia and impaired expression of fear-conditioned analgesia in the stress- and pain-hyperresponsive Wistar-Kyoto rat strain: A role for anandamide in the infralimbic cortex? 10th Neuroscience Ireland Conference, Galway. Poster Presentation
- 3) **Corcoran. L**, Rea. K, Roche M, Finn DP (2016). Impaired Expression Of Fear-Conditioned Analgesia In The Stress- And Pain-Hyperresponsive Wistar-Kyoto Rat Strain. 26th Annual Symposium of the International Cannabinoid Research Society, P2-9 Poster Presentation
- 4) **Corcoran. L**, Rea. K, Roche M, Finn DP (2015). The endocannabinoid system in the anterior cingulate cortex plays a role in the termination of FCA in rats. 9th Neuroscience Ireland Conference, Dublin. Poster Presentation
- 5) **Corcoran. L**, Rea. K, Roche M, Finn DP (2015). The endocannabinoid system in the anterior cingulate cortex plays a role in the termination of FCA in rats. European Cannabinoid Conference 2015, Sestri, Italy. Poster Presentation

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- 2) **Corcoran. L**, Rea. K, Roche M, Finn DP (2017). Hyperalgesia and impaired expression of fear-conditioned analgesia in the stress- and pain-hyperresponsive Wistar-Kyoto rat strain: A role for anandamide in the infralimbic cortex? Centre for Pain Research Day, National University of Ireland, Galway, March 2017. **Oral Presentation (1st Prize)**
- 3) **Corcoran. L**, Rea. K, Roche M, Finn DP (2017). Hyperalgesia and impaired expression of fear-conditioned analgesia in the stress- and pain-hyperresponsive Wistar-Kyoto rat strain: A role for anandamide in the infralimbic cortex? College of Medicine, Nursing

and Health Sciences postgraduate research day, National University of Ireland, Galway.
Poster presentation

- 4) **Corcoran. L**, Rea. K, Roche M, Finn DP (2016). Impaired Expression Of Fear-Conditioned Analgesia In The Stress- And Pain-Hyperresponsive Wistar-Kyoto Rat Strain. Centre for Pain Research Day, National University of Ireland, Galway. **Poster Presentation (1st Prize)**
- 5) **Corcoran. L**, Rea. K, Roche M, Finn DP (2016). Impaired Expression Of Fear-Conditioned Analgesia In The Stress- And Pain-Hyperresponsive Wistar-Kyoto Rat Strain. Irish Pain Society Annual Scientific Meeting, Dublin, Ireland. **Poster (1st Prize)**
- 6) **Corcoran. L**, Rea. K, Roche M, Finn DP (2016). Impaired Expression Of Fear-Conditioned Analgesia In The Stress- And Pain-Hyperresponsive Wistar-Kyoto Rat Strain. College of Medicine, Nursing and Health Sciences postgraduate research day, National University of Ireland, Galway. **Oral Presentation (1st Prize)**
- 7) **Corcoran. L**, Rea. K, Roche M, Finn DP (2015). Impaired Expression Of Fear-Conditioned Analgesia In The Stress- And Pain-Hyperresponsive Wistar-Kyoto Rat Strain. Centre for Pain Research Day, National University of Ireland, Galway. Oral Presentation
- 8) **Corcoran L**, Rea K, Roche M, Finn DP. (2015), The endocannabinoid system in the anterior cingulate cortex mediates fear-conditioned analgesia in rats. College of Medicine, Nursing and Health Sciences postgraduate research day, National University of Ireland, Galway. Poster Presentation.
- 9) **Corcoran L**, Rea K, Roche M, Finn DP. (2014), The endocannabinoid system in the anterior cingulate cortex mediates fear-conditioned analgesia in rats. Centre for Pain Research Day, National University of Ireland, Galway. Poster Presentation.

Chapter 1 : General Introduction

1.1 Introduction

Pain can be defined as ‘an unpleasant sensory and emotional experience associated with actual or potential tissue damage’ (International Association for the Study of Pain [IASP] Task Force on Taxonomy, 1994). Pain can be categorised as either acute or chronic pain. Acute pain is adaptive in nature and can protect an organism from injury or tissue damage (e.g. feeling pain when touching a hot surface). This is exemplified by the fact that those with congenital insensitivity to pain often die early from undetected injuries that are usually signalled via pain (Nagasako et al., 2003). Chronic pain is maladaptive in nature and is usually defined as pain persisting for over 3 months. It may be neuropathic, inflammatory or idiopathic in nature (Aguggia, 2003). Epidemiological studies of 289 diseases and injuries concluded that chronic pain conditions were among the 10 conditions resulting in the longest number of years lived with disability (Vos et al., 2012). Recent data indicate that approximately 20% of the population suffer from chronic pain, the majority of whom also suffer from some other disability or mood disturbance (Blyth et al., 2001, Demyttenaere et al., 2007, Vos et al., 2012).

Under normal circumstances there is a balance of inhibition/facilitation of pain and this is maintained to ensure that only necessary stimuli are perceived by the brain. Pain disorders such as chronic pain occur upon activation of descending facilitation and/or peripheral and central sensitisation. Peripheral sensitisation involves inflammatory mediated increases in the number of ‘silent’ nociceptors becoming ‘active’ and a reduction in pain threshold (Curatolo et al., 2006). Central sensitisation occurs at the level of the dorsal horn of the spinal cord and involves an increase in neuronal excitability leading to abnormal responses and increased activity in response to incoming input from primary afferent neurons including A δ - and C-fibres. In central sensitisation sub-threshold inputs are recruited to nociceptive neurons leading to a reduced activation threshold and an increased response and output (Latremoliere and Woolf, 2009). Descending facilitation will be discussed later in this chapter.

Current pharmacotherapies for pain management lack efficacy in many patients, with ~40% of patients with chronic pain unsatisfied with their treatment (Breivik et al., 2006). Furthermore, the annual economic cost of pain in the US has been estimated at a staggering \$560 - \$625 billion annually, including direct and indirect costs (Gaskin and Richard, 2012, McCarberg and

Billington, 2006, Turk, 2002). The annual cost of treating chronic pain in Ireland is €5.34 billion a year (Raftery et al., 2012). The PRIME study (Prevalence, Impact and Cost of Chronic Pain) revealed a 35.5% prevalence of chronic pain in the Republic of Ireland, with 12% of study's participants either unable to work or limited in the number of hours they could work due to pain (Raftery et al., 2011). Osteoarthritis and rheumatoid arthritis are the most common causes of pain, accounting for 42% of cases, chronic pain from trauma and surgery accounts for 15% while other forms of chronic pain include deteriorated or herniated discs and degeneration or fractures of the spine. The most common areas of pain include the back and joints (unspecified), head, shoulder, neck, knee and leg (Breivik et al., 2006).

The management of pain disorders is necessary to improve the quality of life of patients suffering from them. There are a wide variety of analgesic drugs including anticonvulsants, anti-depressants, local anaesthetics, non-steroidal anti-inflammatory drugs (NSAIDs) and opioid analgesics. However, as already mentioned, many of these drugs lack efficacy and they are also associated with many adverse side effects including addiction, gastrointestinal upset, impaired motor coordination, impaired cognitive performance and sedation (Breivik et al., 2006). Despite the efforts of the research community and the pharmaceutical industry to invest in and develop new drugs to manage pain, chronic pain continues to represent a major unmet clinical need. Thus, further research is needed to understand fully the neurobiological mechanisms of pain, and its modulation, with a view to identifying novel targets and developing new, superior analgesics.

1.2 Neural pathways mediating and modulating pain

Upon exposure to noxious stimuli, either mechanical, thermal or chemical, sensory nociceptive information is relayed via the primary afferent neurons to the dorsal horn of the spinal cord (DHSC). The primary afferent neurons are classified into two types (A- and C-fibres) based on their diameter, structure and conduction velocity. A-fibres are further classified into two types A δ which are medium myelinated fibres and have an intermediate conduction velocity (12–30m/sec⁻¹) and A β which are large myelinated fibres but have a fast conduction velocity (30–100m/sec⁻¹). Under normal circumstances A δ -fibres elicit a rapid sharp type of pain whereas the unmyelinated C-fibres evoke a later long-lasting dull pain (Millan, 1999). Substances such as kinins, nitric oxide, histamine, prostanoids, adenosine and serotonin sensitise these primary afferent neurons (Braszko and Koscielak, 1975, Fox et al., 1974, Hutter and Strein, 1988,

Rosenthal, 1949). Nociceptive information is relayed via the primary afferent neurons to the superficial laminae I and II, and the deeper laminae V, VI and X of the dorsal horn of the spinal cord. These regions are associated with the reception, processing and transmission of this nociceptive information (Millan, 1999). Information is relayed via the second order neurons to the brain via the ascending pain pathways (Willis, 1985a, Willis, 1985b). See Figure 1.1 for a graphical representation of the ascending and descending pain pathways.

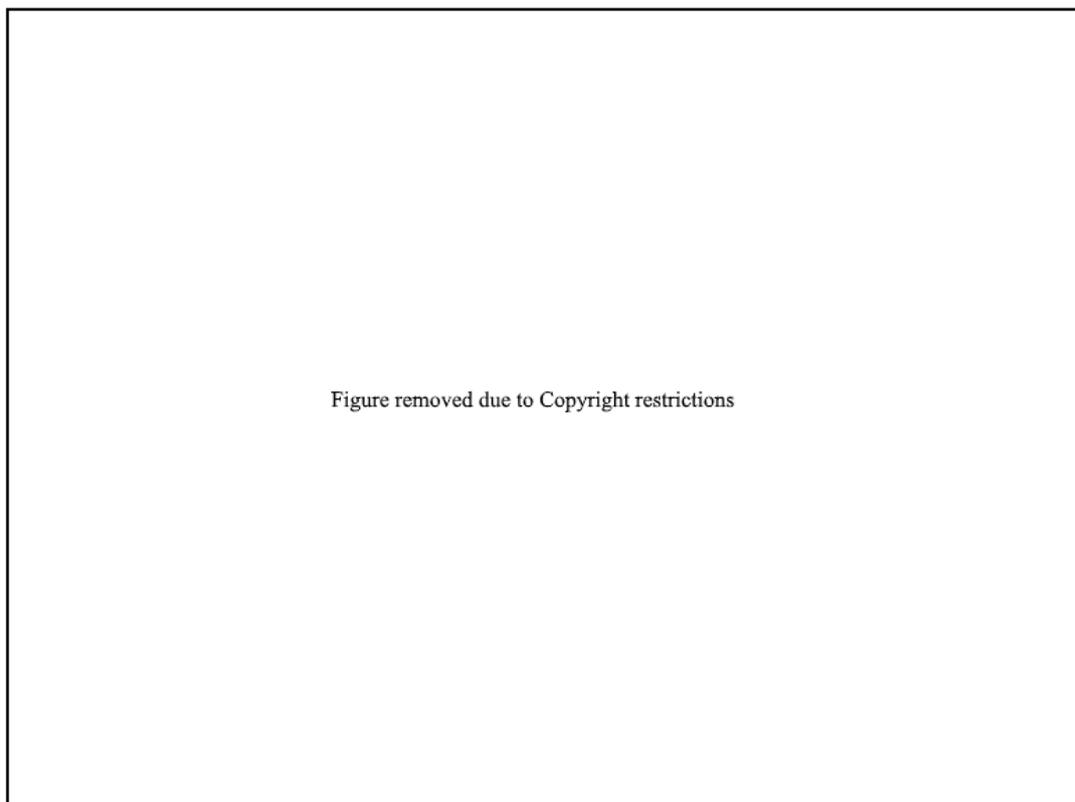


Figure 1.1: Ascending and descending pain pathways; Nociceptive information is relayed via primary afferent neurons to the dorsal horn of the spinal cord. The spinothalamic pathway is important in the sensory-discriminative aspects of pain and projects onto thalamic nuclei where it synapses with third order neurons that terminate in the postcentral gyrus of the cortex. The spinoparabrachial pathway is important in the cognitive-affective aspects of pain and projects to the parabrachial nucleus, from where third order neurons project to the hypothalamus, amygdala and cortex. The descending pain pathway originates in the higher brain regions, such as the cortex, hypothalamus and amygdala. These project onto the PAG and RVM and finally to the dorsal horn of the spinal cord. This pathway can either inhibit or facilitate pain. PAG, periaqueductal grey; RVM, rostral ventromedial medulla; DRG, dorsal root ganglia; PBN, parabrachial nucleus.

1.2.1 Ascending pain pathways

There are multiple ascending pathways including the spinothalamic tract, the spinoparabrachial tract, the spinoreticular tract, the spinomesencephalic tract, and the spinohypothalamic tract. Second order neurons project contralaterally to their respective cerebral targets following decussation of the pathways at the midline (Millan, 1999). See Figure 1.1.

The spinothalamic tract is directly involved in the sensory-discriminative aspects of pain such as intensity, location, duration, temporal pattern and quality. It projects from laminae I, II and IV – VIII of the dorsal horn to the thalamus where the neurons synapse with third order neurons that project to the postcentral gyrus of the cortex (Basbaum et al., 2009, Millan, 1999). The spinothalamic tract can be further categorised into three pathways, the ventral spinothalamic tract, projecting to the lateral complex of the thalamus that functions in the sensory discriminative component of pain, the dorsal spinothalamic pathway, that projects to the nuclei of the posterior medial and intralaminar complexes of the thalamus involved in the affective-motivational aspects of pain, and finally the monosynaptic spinothalamic tract that projects directly to the medial central nucleus of the thalamus involved in the affective component of pain (Almeida et al., 2004, Hodge and Apkarian, 1990, Lovick, 1996, Lumb and Lovick, 1993).

The parabrachial nuclei (PBN) receive direct and indirect inputs via the spinoparabrachial tract and then synapse with third order neurons that relay information to the thalamus, amygdala and hypothalamus. The spinoparabrachial tract has an important role in the cognitive-affective aspects of pain as well as neuroendocrine responses to pain (Basbaum et al., 2009, Derbyshire et al., 1997).

The spinoreticular tract originates in laminae I, V, VII, VIII and X and is involved in the motivational-affective characteristics and the neurovegetative or autonomic responses to pain via its projections to the medial pontobulbar reticular formation (Millan, 1999).

The spinomesencephalic tract plays an important role in the sympathetic and motivational responses to pain via its connection to the spinothalamic tract which projects to the PBN and periaqueductal grey (PAG) via this pathway (Allen and Pronych, 1997, Bernard et al., 1996, Keay et al., 1997).

Neurons from the spinohypothalamic tract respond to noxious stimuli coming from muscles, tendons, joints, skin and viscera and project to the hypothalamus, contributing to the

neuroendocrine, autonomic, motivational-affective and alert responses to somatic and visceral pain (Millan, 1999).

The thalamus represents a key relay point for nociceptive information and is involved in the reception, integration and transfer of this information to the cortex (Millan, 1999, Basbaum et al., 2009). The PAG, PBN, amygdala, striatum, hypothalamus and hippocampus also represent direct and indirect targets of ascending nociceptive inputs as mentioned above. These targets are themselves widely interlinked and participate in the descending modulation of pain described below.

1.2.2 Descending pain pathways

In 1965, Melzack and Wall proposed the now well known ‘Gate Control Theory’. This theory suggested that rather than being automatically transmitted to supraspinal regions, nociceptive information is integrated at the level of the DHSC in a process of inhibitory pain modulation (Melzack and Wall, 1965). This theory has contributed significantly to our understanding of the central mechanisms that modulate pain. The descending control of pain can either impede (descending inhibition) or enhance (descending facilitation) nociceptive transmission (Millan, 2002). The supraspinal structures involved in both descending inhibition and facilitation are not generally mutually exclusive (Millan, 2002). See Figure 1.1.

1.2.2.1 Descending inhibition

Activity in ascending pain pathways can be reduced by supraspinal regions in a process known as descending inhibition. These supraspinal regions play a key role in the modulation of nociceptive transmission within the DHSC (Hopkins and Holstege, 1978, Beitz, 1982). The basolateral amygdala (BLA) projects to the central nucleus of the amygdala (CeA) while also receiving input from higher brain regions such as the prefrontal cortex (Neugebauer et al., 2004). The CeA is a major output nucleus for the PAG (Hopkins and Holstege, 1978, Oka et al., 2008). The PAG projects to the rostral ventromedial medulla (RVM; (Wang et al., 2014b, Morgan et al., 2008)). The RVM can modulate spinal pain transmission through the activity of its ON and OFF cells, with OFF cell activation suppressing pain via descending inhibition (Vanegas et al., 1984, Heinricher et al., 2009). Thus the amygdala, PAG and RVM comprise

major components of the descending inhibitory pain pathway to the DHSC (Basbaum and Fields, 1984).

1.2.2.2 Descending facilitation

Descending facilitation enhances rather than inhibits ascending transmission of nociceptive information from the DHSC (Burgess et al., 2002, Kovelowski et al., 2000, Porreca et al., 2002). Ordinarily, there is a balance between the mechanisms of descending inhibition and descending facilitation. However, in conditions of persistent pain, such as chronic pain, neuroplastic changes lead to an increased facilitatory influence that amplifies the pain experience. Most of the supraspinal structures involved in descending inhibition (amygdala, PAG and RVM) also play a role in mediating descending facilitation (De Felice and Ossipov, 2016, Millan, 1999, Millan, 2002). Of particular importance is the PAG-RVM connection. While OFF cell activity suppresses pain in descending inhibition, ON cell activity enhances pain via interaction with the DHSC in descending facilitation (Vanegas et al., 1984).

1.3 Emotional modulation of pain

1.3.1 Fear/Stress/Anxiety

Fear is a response triggered by direct or imminent threats. It results in rapid defensive responses such as immobility, attack or escape (fight or flight) as well as autonomic and hormonal changes and often feelings of dread and despair (Izquierdo et al., 2016). It is an evolutionarily conserved reaction to dangerous or hazardous situations which threaten to perturb homeostasis, and an important survival strategy (Mineka and Ohman, 2002, Rosen and Schulkin, 1998). Under normal circumstances an appropriate response to a threat is elicited. However, when the response to fear is disproportional or expressed in situations that would not be considered harmful then it becomes maladaptive and a fear or anxiety disorder may result (Marks, 1987).

Unlike fear, anxiety is a long-lasting response that is elicited by potentially dangerous but unspecified future threats (Izquierdo et al., 2016, Lutz et al., 2015). It is an innate behavioural state involving input from multiple senses to assess for potential dangers and initiate appropriate responses. Anxiety-like behaviours include avoidance, decreased motion, increased heart rate and hypervigilance. Under normal circumstances these responses occur at an intensity important for survival. However, when the behaviours become chronic and

disproportionate to the level of danger, anxiety-related neuropsychiatric disorders can occur (Lutz et al., 2015).

The experience of stress is common to all living things and generally refers to a psychological or physical disturbance in homeostasis. Like fear and anxiety, under normal circumstances stress normally triggers a multi-systemic response to restore homeostasis (Herman et al., 2003). When the stress response is either too low or too high, and/or prolonged, an individual is at risk of developing a stress-related affective disorder. Fear usually brings about anxiety and causes stress, but is different from both (Izquierdo et al., 2016).

The intensity and severity of perceived pain does not necessarily correlate with the degree of tissue damage, injury, or inflammation occurring. The importance of context, and modulation of pain by emotion is now widely recognized. Stress, fear, and anxiety exert important modulatory influences on pain (Asmundson and Katz, 2009, Burke et al., 2015, Butler and Finn, 2009, Fitzgibbon et al., 2015, Ford and Finn, 2008, Jennings et al., 2014, Okine et al., 2014, Rhudy and Meagher, 2000, Wiech and Tracey, 2009). Regardless of arousal level, positive emotions generally act to inhibit pain, while negative emotions with low to moderate arousal tend to enhance pain, and negative emotions with high arousal inhibit pain (de Wied and Verbaten, 2001, Dougher, 1979, Meagher et al., 2001, Rhudy and Meagher, 2000, Rhudy and Meagher, 2001, Rhudy and Meagher, 2003a, Rhudy and Meagher, 2003b). Therefore, it is important to consider that the arousal level of an individual may induce hyperalgesia or analgesia in aversive situations.

1.3.2 Stress-induced analgesia - pain suppression by negative affect

1.3.2.1 Stress-induced analgesia / Fear-conditioned analgesia

Historically pain and aversion were studied as two separate and distinct modalities. However, as mentioned above stress/fear/anxiety can profoundly influence pain, either enhancing or inhibiting it in both rodents and humans. The phenomena of fear-conditioned (or fear-induced) and stress-induced analgesia respectively (fear-conditioned analgesia; FCA and stress-induced analgesia; SIA), are now widely studied mechanisms of robust endogenous analgesia. This type of analgesia utilizes the principle of Pavlovian conditioning. Here, conditioning pairs a conditioned stimulus (CS; e.g. a conditioning arena or loud tone) with an unconditioned stimulus (US; e.g. footshock). Following the pairing of both US and CS, re-exposure to the CS alone is enough to elicit behavioural and neuroendocrine responses comparable to those

expressed in the presence of both (Antoniadis and McDonald, 1999). Both CS and US can induce analgesia in the form of FCA or SIA. Exposing an animal to an US (i.e. footshock) can induce a robust fight or flight response and analgesia in the form of SIA (Fanselow, 1984, Grau, 1984, Maier, 1989). Exposure to a CS (i.e. conditioning arena) that was previously paired with an aversive US (i.e. footshock) results in a robust analgesia in the form of FCA (Finn et al., 2004, Helmstetter and Bellgowan, 1993, Iwata and LeDoux, 1988, LeDoux et al., 1988). At their peak, FCA and SIA can suppress pain by as much as 90% (Finn et al., 2004). This form of analgesia is thought to require activation of the descending inhibitory pain pathway. The neurobiology of FCA/SIA will be discussed in further detail later.

Fear is an immediate response to threat or aversion, as described above. In SIA/FCA rats will generally express intense fear-related behaviour in the form of freezing and 22kHz ultrasonic vocalisation and analgesia. Inhibition of pain in this situation is vital because pain-related responses may interfere with and compromise defensive behaviours, such as escape (Bolles, 1980).

Fear/stress/anxiety do not always inhibit pain. As mentioned different levels and types of stress can either exacerbate or inhibit pain. The concept of stress-induced hyperalgesia (SIH) (a robust increase in pain following stress) will be discussed later in this section.

1.3.2.2 Animal models of SIA/FCA

Animal models of FCA involve a combination of one or more stressors paired with one or more pain tests. Preclinical animal models of FCA are essential to understanding the exact mechanisms behind the robust endogenous analgesia it produces. They are also key to revealing possible therapeutic targets for acute and chronic pain as well as comorbid pain and stress related disorders. Butler and Finn (2009) have outlined the numerous different models that are available to study SIA/FCA (Butler and Finn, 2009). A noxious and an aversive stimulus are needed in preclinical animal models (typically rats or mice). The aversive stimulus can be an US or a CS. Unconditioned aversive stimuli (as used in SIA) include footshock, forced swim stress, cold water immersion, infantile isolation, exposure to a novel arena, elevated plus maze, social conflict, or predators such as biting mice, biting flies, cats, or snakes. Conditioned aversive stimuli (as used in FCA) include re-exposure to an arena/ context previously paired with footshock or noxious thermal stimulation. The noxious stimuli that are generally paired

with aversive stimuli include injection of the chemical irritant formalin, radiant heat, insect bites, tail pinches, and intracutaneous electrical current (Butler and Finn, 2009)

1.3.2.3 Human models of SIA/FCA

SIA/FCA can also be modelled in humans, which allows us to understand the impact of stress/fear on pain perception in healthy volunteers. Butler and Finn (2009) have outlined the different human models that are used to study SIA/FCA. In a similar manner to animal models, the aversive stimuli can be either unconditioned or conditioned. US include footshock, handshock, peripheral electrical stimulation, nerve stimulation, exposure to mental arithmetic and noise, hypnosis, and virtual reality video game playing. CS include re-exposure to footshock or handshock, exposing people with spider phobias to spiders, exposing war veterans with post-traumatic stress disorder (PTSD) to videos of combat, re-exposure to auditory stimulus previously paired with mental arithmetic. The noxious stimuli paired with the CS or US in human models include the flexion reflex, radiant heat, intracutaneous electric current, lower back pain, acute burn pain or dental/tooth pain (Butler and Finn, 2009).

Different factors can determine the outcome of stress/fear on pain perception and behaviour. These include genetics, age, experience, gender and the nature of the stressor used (acute or chronic) (Panocka et al., 1986, Ghirardi et al., 1994, Kinsley et al., 1988, Bodnar et al., 1988, Butler and Finn, 2009, Rivat et al., 2007). It is also important to note that modelling SIA/FCA in both animals and humans does not come without some confounding factors. In animal studies subjects are completely unaware of the nature of the stimulus that they are about to receive, while ethical guidelines require that humans are aware they are in a “pain” experiment. Human anticipation of a noxious stimulus as well as the variation of pain perception across individuals may confound the results (Butler and Finn, 2009). Animal models cannot tell us exactly how anxious they are or how much pain they are perceiving in a given situation. This is combated using scoring and formulas to precisely record different pain and fear behaviours. In contrast humans can tell us exactly what they are feeling and when they are feeling it. Although this may give better accuracy when recording pain and fear, it also allows people to lie about how much they are perceiving either to seem stoic or to garner more sympathy.

1.3.2.4 Clinical significance of SIA/FCA

The study of SIA/FCA combines the study of two highly important evolutionarily conserved survival responses in mammals. The ability to activate and utilize a potent intrinsic analgesic mechanism has huge clinical potential. Manipulation of this mechanism can lead to exciting new therapies for pain disorders, although, it is unlikely that SIA/FCA *per se* will ever be used as a clinical treatment. However, if it were possible to engage the same analgesic system without inducing stress/anxiety/fear, then this could be used as a new treatment strategy. Research focusing on the role of the opioidergic system in SIA/FCA in rodents and humans has led to an improved understanding of their role in endogenous analgesia (Willer and Albe-Fessard, 1980, Willer et al., 1981, Pitman et al., 1990, Amit and Galina, 1986, Harris and Westbrook, 1994). Opioids are one of the most widely prescribed drug for pain relief worldwide (Trescot et al., 2008). However, due to their adverse side effect profile and abuse potential new therapeutics options are needed (Kunnumpurath et al., 2018). Understanding the mechanisms underlying SIA/FCA, with a particular focus on the endocannabinoid system (discussed later in this introduction) may offer new therapeutic potential for the treatment of pain disorders.

SIA/FCA could also be used as a potential diagnostic tool for individuals where therapeutic intervention does not offer any pain relief. Impaired expression of SIA/FCA could imply an inability in these individuals to activate or utilize their descending analgesic system (see result chapter 6). It would be interesting to determine if individuals with impaired expression of SIA/FCA are also predisposed to develop chronic pain or co-morbid pain and stress disorders. This could be done in a manner similar to conditioned pain modulation studies in humans to evaluate an individual's capabilities to inhibit pain (Kennedy et al., 2016).

Indeed, distraction-induced analgesia involves distracting rather than aversive stimuli to induce analgesia in subjects. Some forms of SIA/FCA may have a distraction component to them (Hoffman et al., 2001, Schmitt et al., 2011). Thus, it can be said that fear is sufficient but not necessary for conditional SIA/FCA (Butler and Finn, 2009, Helmstetter and Fanselow, 1987, Roche et al., 2007, Roche et al., 2010, Rea et al., 2014b, Kinscheck et al., 1984). Inducing this form of analgesia may also offer relief to those patients where conventional therapeutics offer no alleviation to their pain. If we can unravel the mechanism behind this form of analgesia it may allow us to combine both psychological and medicinal therapy for individuals with acute and chronic pain, thereby reducing the incidence of unwanted side effects, tolerance or addiction in patients.

1.3.2.5 Neurobiology of fear and FCA

As FCA is a major focus of this thesis I will briefly discuss its neurobiology. As previously mentioned, at the supraspinal level pain can either be enhanced (descending facilitation) or reduced (descending inhibition). Pain, fear, stress and anxiety are mediated and modulated by complex and diffuse networks rather than any single region or connection. FCA is believed to be mediated by activation of the descending inhibitory pain pathway (Figure 1.1). Various neural substrates (e.g. prefrontal cortex (PFC), amygdala, PAG, RVM) that are important in the descending inhibitory pain pathway are also key components of the fear/stress/anxiety neurocircuitry.

The medial prefrontal cortex (mPFC) is strongly implicated in cognitive, emotional and motivational processes, and in the regulation of responses to aversion (Guimaraes et al., 2011, Laviolette et al., 2005, Baeg et al., 2001, Jiang et al., 2014, Ji et al., 2010) and pain (Okine et al., 2014, Luongo et al., 2013, Okine et al., 2016, Ji and Neugebauer, 2014, Ji and Neugebauer, 2011). The mPFC has also been implicated in the recall and extinction of fear-related memory of noxious stimuli. The infralimbic cortex (IL) and prelimbic cortex (PrL), subregions of the mPFC, have been shown to differentially affect acquisition, consolidation and expression of contextually conditioned fear (Corcoran and Quirk, 2007, Sharpe and Killcross, 2014, Vidal-Gonzalez et al., 2006, Almada et al., 2015, Sierra-Mercado et al., 2011) as well as impact FCA (Rea et al., 2018). The PrL is known to play a key role in the production of fear while the IL is involved in the extinction of fear (Corcoran and Quirk, 2007, Sierra-Mercado et al., 2011, Laurent and Westbrook, 2009, Vidal-Gonzalez et al., 2006). The anterior cingulate cortex (ACC) is a third subregion of the mPFC and is involved in the cognitive-affective component of pain and fear and in the top-down descending modulation of pain (Calejesan et al., 2000, Fuchs et al., 2014). The ACC has strong reciprocal connectivity with both the PrL and IL may play a role in modulating their output (Vertes, 2002). The mPFC has been shown to project to other neural substrates important in fear and pain neurocircuitry (Hoover and Vertes, 2007), such as the amygdala (Ji and Neugebauer, 2014, Ji et al., 2010, Laviolette and Grace, 2006, Garcia et al., 1999), hippocampus (Jin and Maren, 2015, Marek et al., 2018, Wang et al., 2016) and PAG (LeDoux, 2000, Marchand and Hagino, 1983).

The PrL and IL project downstream to the BLA and intercalated cell mass (ITC) of the amygdala, which then project on to the CeA (Arruda-Carvalho and Clem, 2015a, Little and Carter, 2013, McDonald et al., 1996, Cho et al., 2013). The amygdala has been shown to play a key role in the induction, processing and extinction of conditioned fear (Davis et al., 1994,

LeDoux, 2000, Marsicano et al., 2002). Conditioned and unconditioned stimuli are processed within the BLA and CeA and this information is sent to other brain regions (Izquierdo et al., 2016). Key fear behaviours studied in FCA/SIA studies have been linked to neuronal activity within the amygdala. Both freezing and 22kHz ultrasonic vocalisation can be attenuated upon lesion or pharmacological blockade within the amygdala (Helmstetter, 1992, Helmstetter and Bellgowan, 1993, Maren et al., 1996). The affective dimension of nociception is also processed within the amygdala (Butler et al., 2017, Veinante et al., 2013). Indeed, early lesion studies of the amygdala have demonstrated a reduction in FCA and SIA (Helmstetter, 1992, Watkins et al., 1993, Werka, 1997, Werka, 1994, Werka and Marek, 1990) showing a key role for this region in these processes. A recent study has shown that different neuronal phenotypes and neural circuits between, within and from various amygdala nuclei are involved in the processing of conditioned contextual aversion, nociception and FCA (Butler et al., 2017).

The amygdala has reciprocal connections with the hippocampus (O'Donnell and Grace, 1995, Pikkarainen et al., 1999). The hippocampus plays a key role in the acquisition and memory of conditioned fear (Izquierdo et al., 2016). A recent optogenetic study has shown that neuronal projections from the ventral hippocampus (VH) to different subnuclei of the amygdala play a key role in contextual fear conditioning. VH afferents contact both excitatory principal neurons and local GABAergic interneurons in the BLA and glutamatergic excitatory inputs onto CEA neurons (Cembrowski and Spruston, 2016, Xu et al., 2016). Very few studies have examined the role of the hippocampus in SIA/FCA despite its role in both pain and fear. The amygdala also has important reciprocal connections with the PAG which also plays a very important role in pain, fear and FCA. Like the amygdala, the PAG plays a key role in the expression of both freezing and 22kHz ultrasonic vocalisation and in coordinating defensive and aversive responses to fear and stress (Kim et al., 2013, Kim et al., 1993, De Oca et al., 1998). PAG lesion attenuates FCA and SIA in adult and neonatal rats respectively (Wiedenmayer et al., 2000, Helmstetter and Tershner, 1994).

The PAG projects to the hypothalamus (Behbehani et al., 1988) and RVM (Morgan et al., 2008) which respectively play a key roles in the descending emotional motor system and expression of the cardiac, vascular and somatic motor components of conditioned fear (Furlong and Carrive, 2007, Vianna et al., 2008, Holstege et al., 1996) and descending inhibition and facilitation of pain (Millan, 1999, Millan, 2002, Basbaum and Fields, 1984). Lesions of the hypothalamic arcuate nucleus, ventromedial nucleus and paraventricular nucleus have been shown to attenuate SIA in rats and mice respectively (Millan et al., 1980, Kelsey et al., 1986,

Truesdell and Bodnar, 1987). Lesions of the RVM have been linked to a potentiation of SIA in the formalin test in rats (Shamsizadeh et al., 2014).

The overlap in the neural substrates and circuitry involved in modulating pain and fear is also mirrored by overlap in the neurochemicals regulating these processes. Important roles for gamma-aminobutyric acid (GABA), glutamate, monoamines (serotonin (5HT), dopamine and noradrenaline), opioids and the endogenous cannabinoid (endocannabinoid) system have been described. The endocannabinoid system is a major focus of the research presented within this thesis and will be discussed in more detail later (section 1.4). For an in depth look at other neurochemical systems see (Millan, 2003, Butler and Finn, 2009).

1.3.3 Stress-induced hyperalgesia - pain exacerbation by negative affect

1.3.3.1 Stress-induced hyperalgesia

I have already discussed the effect of robust, intense stress on pain processing (SIA/FCA). This section will focus on the impact of repeated chronic exposure to stress which leads to the less well-understood phenomenon of stress-induced hyperalgesia (SIH). For review see Jennings et al., (2014) and Olango & Finn (2014). SIH is an exacerbation of pain upon exposure to stress. It is now well documented that a high number of patients exhibit co-morbid chronic pain and psychiatric disorders such as anxiety or depression. It is difficult to determine whether pain disorders lead to psychiatric disorders or *vice versa*. However, studies have shown that the negative effects of each of these disorders alone are amplified when they are co-occurring. The comorbid expression of these disorders in a clinical setting often complicates the treatment of either alone, leading to poorer outcomes for patients (Asmundson and Katz, 2009, Bair et al., 2003, Lieb et al., 2007). These data suggest that a reciprocal relationship between chronic pain and affective disorders exists.

While SIA/FCA constitute an important survival response in mammals, the biological role of SIH in terms of survival is less clear. It is possible that the manifestation of hyperalgesia occurs to ensure that mammals seek treatment or rest. Increased understanding of the interactions between the neural substrates and neurochemical mediators that regulate pain and stress is necessary to tease out the mechanisms underlying SIH and produce successful treatment outcomes for patients.

1.3.3.2 Animal models of SIH

Preclinical animal models of SIH are well-studied and employ a number of different aversive stimuli or environments. They are essential for understanding the mechanisms behind this form of hyperalgesia and are key to revealing possible therapeutic targets for pain and its exacerbation by negative affect. Animal models of SIH generally involve repeated or persistent application of a stressor for several days to weeks (chronic stress), in combination with a noxious stimulus. Detailed descriptions of these tests can be found in Jennings et al., (2014). Examples of stressors include forced swim-stress, repeated cold stress, water avoidance stress, immobilisation stress, social defeat, chronic mild stress, rotation stress, maternal separation/deprivation or early life stress, noise stress, vibration stress, whisker pad stimulation, air puff stress, genetic models/strains exhibiting stress-hyper responsivity or anxiodepressive phenotypes, and the single prolonged stress model of PTSD. Noxious stimuli can include, the formalin test, hot plate test, carrageenan injection, tail flick/immersion test, Randall-Sellito apparatus, footshock, von Frey, acetone-induced cold, colorectal distension and thermal escape tests (Jennings et al., 2014).

1.3.3.3 Human models of SIH

Unlike the preclinical animal models of SIH, there are only a small number of human models developed for the study of SIH. These models use either psychological or physiological stressors paired with a noxious stimulus. Psychological models attempt to evoke a state of anticipatory anxiety and its influence on subsequent noxious stimuli. Examples of psychological stress-based models include event-related factorial pain paradigms, threat of electric shock and radiant heat, pre-exposure to experimental psychological stress paired with the cold pressor test and psychosocial stress test paired with thermal stimuli. Physiological models employ stressors to disrupt homeostasis and test its influence on subsequent noxious stimuli. Physiological stress-based models include metyrapone-induced hypocortisolism with mechanical pain sensitivity, and the induction of hypoglycaemia with thermal pain sensitivity (Jennings et al., 2014).

In both humans and rodents, different factors can determine the outcome of stress/fear on pain perception and behaviour. These include genetics, age, environment, gender and the nature of the stressor used (acute or chronic). Like in models of SIA/FCA, there are many confounding factors in models of SIH. In animal studies subjects are completely unaware of the nature of

the stimulus that they are about to receive, while ethical guidelines require that humans are aware they are in a “pain” experiment. Human anticipation of a noxious stimulus as well as the variation of pain perception across individuals may confound the results.

1.3.3.4 Clinical significance of SIH

Pain and stress-related disorders tend to be expressed co-morbidly. Stress, anxiety and depression can influence the perception of pain. Clinically, anxiety is often accompanied by chronic pain complaints. For example, PTSD is frequently accompanied by acute pain episodes as well as chronic musculoskeletal pain (Asmundson, 2002). Similar occurrences can be seen in patients that suffer from chronic pain. The prevalence of clinical anxiety (generalised anxiety disorder, obsessive compulsive disorder, PTSD etc) in chronic pain patients can reach as high as 77% (Knaster et al., 2012). As previously mentioned the co-occurrence of these disorders complicates treatment. The close relationship between anxiety and pain needs to be further elucidated to establish and develop new effective treatments. Very little is known regarding the neurochemical and molecular mechanisms underpinning this co-morbid expression of pain and aversion. The study of SIH can allow us to develop a deeper understanding of the relationship between pain and stress and develop improved treatment options for patients.

1.3.3.5 Wistar-Kyoto rat model – genetic model of hyperalgesia associated with negative affective state

An important factor to consider when studying the influence of negative affect on hyperalgesia is genetics. The Wistar-Kyoto (WKY) rat strain is an in-bred strain with an anxiety- and depressive-like phenotype that is hypersensitive to both stress and pain. The WKY rat strain was first developed from the Wistar rat in 1963 by Okamoto and Aoki as a normotensive control strain for the spontaneously hypertensive rat strain (Okamoto and Aoki, 1963). In-bred rat strains should in theory be genetically identical within each strain thus, using an in-bred strain such as the WKY rat allows a researcher to dissociate genetic and environmental components of a trait. The WKY rat is a useful model for studying the neurobiology underpinning high trait anxiety and depression, and their tendency to exacerbate, or co-occur with, pain states.

WKY rats exhibit aberrant neurochemical and stress responses compared to outbred rat strains. The WKY rat also displays increased stress vulnerability, exhibiting exaggerated

hypothalamic–pituitary–adrenal axis (HPAA) responses to stress with sustained corticosterone response and enhanced plasma adrenocorticotropic hormone response compared with other rat strains (Redei et al., 1994, De La Garza and Mahoney, 2004, Malkesman et al., 2006, Pare and Redei, 1993, Rittenhouse et al.). WKY rats also display greater sensitivity to cholinomimetics due to a low levels and activity of butyrylcholinesterase, compared with Sprague-Dawley (SD) rats (Beck et al., 2001, Servatius et al., 1998). The nucleus accumbens and PFC, regions implicated in anxiety and depression, have altered turnover of serotonin, noradrenaline, dopamine and their metabolites in WKY rats compared to out-bred strains, such as the Wistar and SD rat (De La Garza and Mahoney, 2004, Ferguson et al., 2003, Scholl et al., 2010, Pardon et al., 2003).

WKY rats display increased behavioural inhibition in a variety of situations, for example WKY rats display increased immobility and increased latency to leave the centre of the open field test compared to SD rats (Drolet et al., 2002, Ferguson and Cada, 2003, Nosek et al., 2008). Both male and female WKY rats display an increased acoustic startle response compared to SD rats and other inbred and outbred strains (Glowa and Hansen, 1994, McAuley et al., 2009, Servatius et al., 1998). Acute stress has been shown to increase freezing and reduce activity in the open field test and elevated plus maze in WKY rats (Nosek et al., 2008). In the forced swim test, WKY rats display higher levels of behavioural despair, as evidenced by fewer struggling responses and more floating/immobility behaviour, compared to other strains. This is a sign of depression-like behaviour in rodents (Malkesman and Weller, 2009, Pare, 1992, Pare and Redei, 1993). WKY susceptibility to stress is also evident in their enhanced susceptibility to develop stress-induced ulcers compared to outbred strains (Pare, 1989a, Pare, 1989b, Pare and Schimmel, 1986).

As previously mentioned, the WKY rat displays a hyperalgesic phenotype. This makes it a useful model to study the influence of genetic background on pain modulation during negative affect. WKY rats exhibit thermal hyperalgesia in the hot plate but not tail flick test compared to SD rats (Burke et al., 2010). WKY rats also display hyperalgesia following visceral stimulation (O'Mahony et al., 2010, Greenwood-Van Meerveld et al., 2005, Gunter et al., 2000). WKY rats are sensitive to inflammatory pain, and display enhanced nociceptive behaviour following intra-plantar injection of formalin or complete Freund's adjuvant (CFA) into the paw or temporomandibular joint (Burke et al., 2010, Rea et al., 2014b, Madasu et al., 2016, Wang et al., 2012). Repeated forced swim stress can reduce formalin-evoked nociceptive behaviour in WKY rats compared to SD rats (Jennings et al., 2015).

Understanding the genetic components behind a behaviour plays a key role in understanding psychiatric disorders. Quantitative trait loci (QTL) analysis of genetic loci linked to behaviour in the forced swim test (FST) has unveiled several loci commonly linked to the FST and other published QTL data for anxiety and emotionality measures in mice as well as those associated with human anxiety, major depression or bipolar disorder. These include loci in WKY rats for climbing, immobility and swimming, behavioural characteristics tested in anxiety and stress paradigms, (Imm1 D2Rat188, Imm3 D5Rat40, Imm6 D16Arb5, Climb2 D1Rat147, FST1 D16Rat75) (Solberg et al., 2004). Microarray analysis revealed gene expression differences in 66 genes of the locus coeruleus and 52 in the dorsal raphe between WKY and SD rats (Pearson et al., 2006). The locus coeruleus is an important source of noradrenergic neurons, and genes encoding the enzymes required for noradrenaline synthesis and metabolism had higher expression levels in WKY rats. Dysfunction in the noradrenaline turnover in this region may underlie the stress and depressive phenotype of WKY rats. Expression of genes encoding the cannabinoid type 1 (CB₁) receptor and κ -opioid receptor were increased in the locus coeruleus of WKY rats compared to SD rats. In the dorsal raphe nucleus, genes encoding cytoskeletal proteins for structural plasticity and potassium channels showed reduced expression in WKY rats, compared with SD counterparts (Pearson et al., 2006). The mRNA of catechol-O-methyltransferase (COMT), was expressed at levels four to sevenfold higher in the cerebral cortex of WKY rats compared to SD rats. This enzyme plays a key role in the catabolism of catecholamines and may underlie the depressive-like phenotype of WKY rats (Walker et al., 2004). It is possible that a dysfunction in the monoaminergic system of this rat strain may underlie its genetic predisposition to psychiatric disorders. Immunoblot analysis has revealed significantly higher levels of the fatty acid amide hydrolase (FAAH) enzyme in the frontal cortex and hippocampus of WKY rats compared to Wistar rats. FAAH is the primary catabolic enzyme for the endocannabinoid anandamide (AEA), and related *N*-acylethanolamines. CB₁ expression was significantly increased and AEA and brain-derived neurotrophic factor (BDNF) levels were decreased in the frontal cortex and hippocampus of WKY rats compared to Wistar rats. Chronic FAAH inhibition produced an antidepressant-like effect in WKY rats in the form of decreased immobility in the FST and increased sucrose consumption. FAAH inhibition also elevated BDNF levels in the frontal cortex and hippocampus of WKY compared to vehicle (VEH) treated WKY rats, an effect accompanied by increased AEA levels and decreased CB₁ receptor activity. This study suggests that there may be a critical role for the endocannabinoid system in the genetic predisposition in WKY rats to depressive-like behaviour (Vinod et al., 2012).

It must be noted that although genetic homogeneity is assumed among inbred WKY strains, recent studies have determined that this may not be the case between WKY rats from different suppliers. Genetic heterogeneity in WKY sub-strains from different suppliers may be confounding results and may account for behavioural and neurochemical differences seen across similar studies. In a study by Zhang-James et al., 2013, WKY rat strains from different breeding institutions had heterozygous single nucleotide polymorphisms (SNPs) (Zhang-James et al., 2013). Between any two breeding sources there were significant genetic differences (between 0.11% and 2.64%) present between the SNPs in the sub-strains (e.g. WKY rats from Charles River had 2.64% SNPs different to WKY rats from Harlan) (Zhang-James et al., 2013). Sagvolden et al. analysed WKY rats from both Charles River, Germany and Harlan (now Envigo), UK. As well as finding behavioural differences in impulsivity and attention, they also found that 33.5% of the genome differed with large stretches of divergence on every chromosome they analysed (chromosomes 1, 2 and 3), in the form of microsatellite polymorphisms and SNPs differences (Sagvolden et al., 2008). Browne et al., have demonstrated that WKY lines from Charles River and Harlan laboratories exhibit high baseline immobility in the forced swim test, an effect not seen in WKY rats from the supplier Taconic (Browne et al., 2015). In addition, WKY rats from Charles River developed more ulcers after water avoidance stress than WKY rats from Harlan (Pare and Kluczynski, 1997). It is possible that the reason for the genetic and behavioural divergence seen in WKY strains is due to genetic drift as a result of its breeding history. WKY rats bred in Kyoto School of Medicine were sent to the National Institute of Health (NIH), prior to being fully inbred. Indeed, Charles River laboratories received WKY rats from the NIH in 1971 while Harlan received them much later in 1982. These studies highlight the importance of choosing a supplier and indeed continuing with the same supplier when comparing across studies, although rats from different suppliers are often used equally and interchangeably (Zhang-James et al., 2013).

1.4 The endocannabinoid system

Cannabis sativa, a cultivated plant, was one of the earliest grown by man. Evidence from China suggests the plant was first used there as early as 4000 BC as a fiber plant, its stems (hemp) used to manufacture ropes, textiles and paper, some of which were found in the tomb of Emperor Wu of the Han dynasty (104-87 B.C.) (Li, 1974, Zuardi, 2006). There is evidence of the seeds being used as food *circa* 6000 B.C (Zuardi, 2006, Touw, 1981).

The use of *Cannabis sativa* as a Chinese medicine was first reported in the oldest pharmacopoeia, the pen-ts'ao ching. The Chinese found its medicinal properties useful in the treatment of rheumatic pain, constipation and malaria, among others. It was also used as an anaesthetic during surgical operations (Li, 1974, Zuardi, 2006). The medical properties of *Cannabis sativa* were reported in India around 1000 B.C., where it was recommended as an analgesic, anticonvulsant, anxiolytic and anti-inflammatory agent. The introduction of cannabis to Western medicine came from Irishman W.B. O'Shaughnessy who recorded its analgesic properties and its use in the treatment of rheumatism, convulsions, tetanus and rabies (Russo, 2017).

Although the medicinal properties of the *Cannabis sativa* plant have been known for millennia, it wasn't until the mid to late nineteenth century that its therapeutic potential was examined scientifically (Szaflarski and Bebin, 2014). The discovery of Δ^9 -tetrahydrocannabinol (THC), the major psychoactive component of the plant *Cannabis sativa* in the 1960s (Mechoulam and Gaoni, 1967) led to extensive studies that have revealed the mechanisms underlying the pharmacological and physiological effects of cannabinoids and the endocannabinoid system, respectively.

A significant milestone in cannabinoid research came with the molecular identification of two cannabinoid G-protein coupled receptors (GPCRs): CB₁ receptors (Devane et al., 1988, Matsuda et al., 1990) and cannabinoid type 2 (CB₂) receptors (Munro et al., 1993).

1.4.1 Distribution of CB₁ and CB₂ receptors.

The cannabinoid receptors in the adult human brain and spinal cord are distributed in a heterogeneous fashion (Glass et al., 1997). There are also high densities of cannabinoid receptors in the rodent brain and spinal cord. Quantitative receptor autoradiography and *in-situ* hybridization of CB₁ receptors found high concentrations in the cerebral cortex, basal ganglia, cerebellum and hippocampus (Glass et al., 1997, Herkenham, 1991, Herkenham et al., 1991). The presence of CB₁ receptors in these brain regions likely account for the motor and sensory as well as psychoactive effects of THC. Lower expression was found in the amygdala, brainstem, mid-brain, PAG, hypothalamus and the dorsal horn of the spinal cord (Herkenham, 1991, Herkenham et al., 1991, Glass et al., 1997). CB₁ receptors are also expressed in non-neuronal tissue included the heart, lungs, liver, adrenal gland, pancreas, bone marrow, tonsils,

spleen, glial cells, among others (Bouaboula et al., 1993, Galiegue et al., 1995, Cota, 2007, Cota et al., 2003, Osei-Hyiaman et al., 2005, Cavuoto et al., 2007).

CB₂ receptors, although expressed in the central nervous system (CNS) such as the cerebral cortex, brain stem, hippocampus, amygdala and cerebellum among others (Baek et al., 2008, Concannon et al., 2015, Onaivi et al., 2006, Van Sickle et al., 2005, Zhang et al., 2014) are mainly distributed in the periphery with a particularly high density on cells and tissues of the immune system such as the spleen, tonsils, testis, peripheral blood leukocytes and monocytic cell lines (Bouaboula et al., 1993, Berdyshev, 2000, Munro et al., 1993, Sugiura et al., 1995). The exact function of the CB₂ receptor in the brain has yet to be characterized as thoroughly as the CB₁ receptor.

Following the characterization of the cannabinoid receptors a number of endogenous ligands for the receptors were identified, including arachidonyl ethanolamide (anandamide, AEA) and 2-arachidonoyl glycerol (2-AG) (Devane et al., 1992, Mechoulam et al., 1995, Sugiura et al., 1995). These are the two best characterized endocannabinoids. Other less well-studied endocannabinoid ligands include oleamide (Leggett et al., 2004), O-arachidonoyl ethanolamine (Virodhamine) (Porter et al., 2002), 2-arachidonoyl glycerol ether (noladin ether) (Hanus et al., 2001) and N-arachidonoyl-dopamine (NADA) (Huang et al., 2001, Bisogno et al., 2005). The n-acylethanolamines, N-oleoylethanolamide (OEA) and N-palmitoyl-ethanolamide (PEA) derived from fatty acids are two metabolically related AEA analogs and endogenous ligands within the endocannabinoid system.

1.4.2 Other receptor targets for the endocannabinoids

In addition to the two classical cannabinoid receptors (CB₁ and CB₂), several lines of evidence suggest a role for the endocannabinoids acting at numerous other sites such as the transient receptor potential vanilloid 1 (TRPV1), members of the nuclear receptor family, peroxisome proliferator-activated receptors (PPARs, with three subtypes α , β (δ) and γ), and the G protein-coupled receptors GPR55 and GPR119 (Brown, 2007, O'Sullivan, 2007, Alexander and Kendall, 2007).

TRPV1 is a non-selective transmitter-gated ion channel widely distributed throughout the brain (Toth et al., 2005). In the peripheral nervous system it is expressed on small diameter primary afferent fibres, acting as a focal point for the summation of noxious stimuli such as high temperature and low pH (Alexander and Kendall, 2007). Anandamide has been shown to be a

full agonist at TRPV1 despite only being a partial agonist at CB₁ (Smart et al., 2000, Zygmunt et al., 1999). Under certain conditions, such as inflammation or cell damage, AEA may exert its effects via TRPV1 rather than CB₁ resulting in contrasting effects in pain behaviour and inflammation (Di Marzo et al., 2002).

The PPARs belong to a family of nuclear receptors consisting of three isoforms α , β (δ) and γ . Following heterodimerization with the retinoid X receptor they bind to DNA sequences called PPAR response elements leading to transcription of gene targets upon ligand activation (O'Sullivan, 2007). They are widely expressed in both peripheral and CNS tissue (Braissant et al., 1996, Moreno et al., 2004). PPARs play an important role in the regulation of metabolism, energy homeostasis, inflammation, pain, insulin sensitivity and food intake (Ferre, 2004, Delerive et al., 2001, Glass and Ogawa, 2006, Stienstra et al., 2007). Evidence now suggests that the endocannabinoids are natural activators of the PPARs. PPARs are activated by a range of compounds including PEA, OEA, noladin ether and virodhamine (Sun and Bennett, 2007). They are also activated by a range of synthetic cannabinoids, along with cannabinoid antagonists (O'Sullivan, 2007). In addition, evidence suggests that 2-AG-induced suppression of the cytokine IL-2 is mediated through PPAR γ (Rockwell et al., 2006).

GPR55 and GPR119 are two orphan G protein-coupled receptors that have recently been implicated as novel cannabinoid receptors. GPR119 has been proposed as a receptor for OEA while GPR55 has been proposed as a receptor for multiple different endocannabinoid ligands including AEA, 2-AG, PEA and OEA (Brown, 2007, Ryberg et al., 2007). Further studies are required to determine the functional significance of endocannabinoid modulation of this receptor.

1.4.3 Endocannabinoid biosynthesis

Endocannabinoids are not stored in vesicles and so their biosynthesis occurs on demand (Di Marzo and Deutsch, 1998). Cannabinoid receptors are activated via retrograde transmission (Figure 1.5), therefore at a neuronal level, AEA and 2-AG are synthesized in the postsynaptic neurons, released into the synaptic cleft, and bind to CB₁ receptors expressed on presynaptic nerve terminals (Kreitzer and Regehr, 2002, Wilson and Nicoll, 2001). All cells and tissues in the body possess the enzymes responsible for the production of AEA and 2-AG including the neurons in the CNS, oligodendrocytes (Gomez et al., 2010), astrocytes and microglia (Hegyí et al., 2012).

1.4.4 Anandamide biosynthesis

AEA is synthesized through a two-step process from the membrane phospholipid precursor n-acylphosphatidylethanolamine (NAPE). In the first step, NAPE is produced by N-acyltransferase (NAT) when a calcium-dependent transacylase catalyses the transfer of arachidonic acid from phosphatidyl choline (PC) to phosphatidylethanolamine (PE) (Di Marzo and Deutsch, 1998, Di Marzo et al., 1994). In the second step, NAPE is hydrolysed to AEA and phosphatidic acid via N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD). Evidence suggest the existence of two additional pathways involved in the synthesis of AEA. The first involves deacylation of NAPE by α,β -hydrolase 4 (Abhd4) and the subsequent cleavage of glycerophosphate to yield AEA, and the second involves PLC-mediated hydrolysis of NAPE to yield phosphoanandamide which is then dephosphorylated to AEA (Di Marzo and Deutsch, 1998). See Figure 1.2.

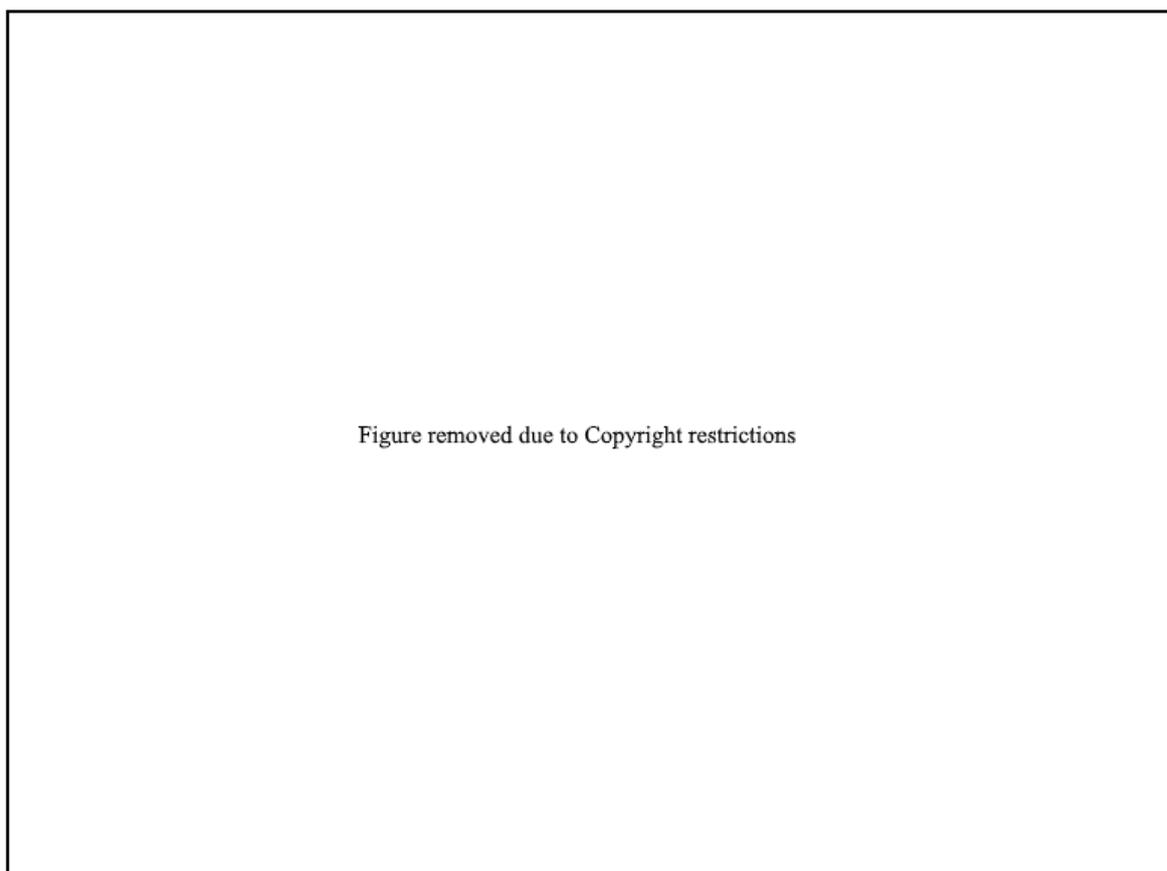


Figure 1.2: Major pathway for synthesis of AEA; (Fonseca et al., 2013).

1.4.5 2-Arachidonoylglycerol biosynthesis

2-AG is synthesized following the cleavage of the membrane phospholipid phosphatidylinositol (PI) by phospholipase C (PLC) to yield 1,2-diacylglycerol (DAG). DAG is then converted to 2-AG by the enzyme diacylglycerol lipase (DAGL) (Fonseca et al., 2013, Prescott and Majerus, 1983, Sugiura et al., 1995).

An alternative pathway for the biosynthesis of 2-AG has been proposed which suggests that phospholipase A1 (PLA1) may cause the hydrolysis of PI to an intermediate 2-arachidonyl-lysophospholipid which is converted to 2-AG via lyso-phospholipase C (lyso-PLC) activities (Sugiura et al., 1995, Fonseca et al., 2013). See Figure 1.3.

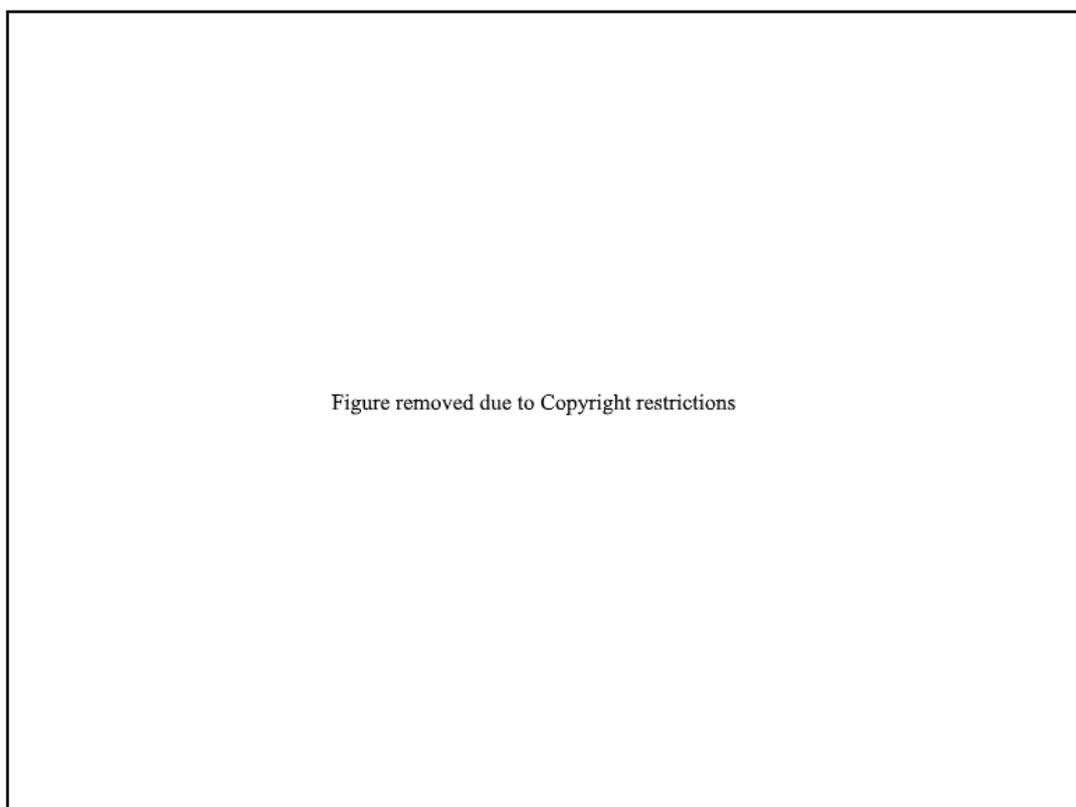


Figure 1.3: Major pathway for synthesis of 2-arachidonoylglycerol; (Fonseca et al., 2013).

1.4.6 N-Acylethanolamines

AEA production is often accompanied by high amounts of N-acylethanolamides such as OEA and PEA derived from fatty acids. These are biosynthesized in a similar manner to AEA using the fatty acids palmitic acid and oleic acid respectively. Both are bioactive, with PEA acting as

an antinociceptive and anti-inflammatory agent, and OEA regulating feeding and body weight (Ho et al., 2008, Rea et al., 2007). PEA and OEA, unlike AEA, have little agonist activity at the CB₁ and CB₂ receptors and have the ability to activate non-CB receptors such as TRPV1, PPARs and GPR55 (Ho et al., 2008, Lin et al., 1998, Vandevoorde et al., 2003, Lambert et al., 1999, Griffin et al., 2000a, Alexander and Kendall, 2007, Sheskin et al., 1997). They may potentiate the effect of AEA on cannabinoid or vanilloid receptors in what is described as an “entourage effect”. This is mediated by competitive inhibition of AEA hydrolysis by FAAH (Ho et al., 2008, Mechoulam et al., 1998, De Petrocellis et al., 2001b).

1.4.7 Endocannabinoid signalling in neurons

CB₁ and CB₂ receptors are Gi/o protein-coupled receptors negatively coupled to adenylate cyclase (AC), (Howlett, 1985, Howlett et al., 1999) and positively coupled to mitogen-activated protein kinase (MAPK) (Bouaboula et al., 1995). See Figure 1.4.

Therefore, activation of the cannabinoid receptors activates Gi/o proteins (Pertwee, 1997) resulting in the inhibition of AC and associated cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) levels (Wade et al., 2004), in addition to stimulation of MAPK (Hoffman and Lupica, 2000). This leads to major changes in cellular activity including gene expression (Bouaboula et al., 1995). Activation of the CB₁ and CB₂ receptors leads to the expression of immediate early genes such as *zif268*, *c-Fos* (Patel et al., 1998) and *c-Jun* N-terminal kinase (JNK1 and JNK2).

Cannabinoid receptors also couple to ion channels through their Gi/o proteins. Cannabinoid receptors are coupled positively to type A channels and inwardly rectifying potassium channels and produce a concentration-related inhibition of voltage-gated L, N, and P/Q inward calcium currents (Pertwee, 1997). The activation of the inwardly rectifying potassium channels inhibits neurotransmitter release (McAllister et al., 1999) including noradrenaline (Kathmann et al., 1999, Göbel et al., 2000), dopamine (Kathmann et al., 1999, Cadogan et al., 1997), 5HT (Nakazi et al., 2000), GABA (Vaughan et al., 1999, Irving et al., 2000, Takahashi and Linden, 2000) and glutamate (Auclair et al., 2000, Vaughan et al., 2000).

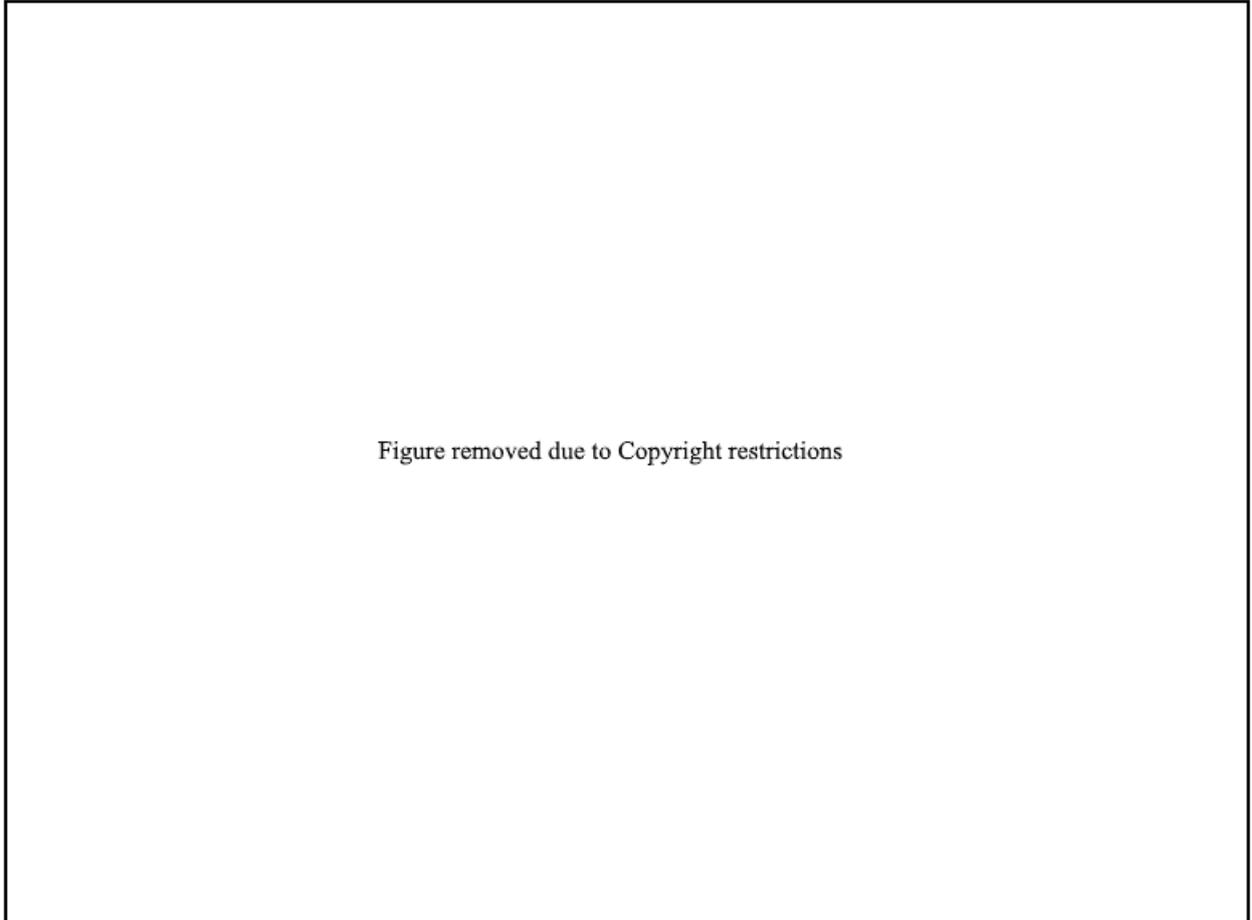


Figure 1.4: Major signalling pathways upon endocannabinoid receptor activation. AC, adenylate cyclase; PKA, protein kinase A; MAPK, mitogen-activated protein kinase; ATP adenosinetriphosphate (Di Marzo et al., 2004).

1.4.8 Endocannabinoid transport and uptake

The mechanisms underlying endocannabinoid biosynthesis, signalling and degradation are relatively well known but there is controversy surrounding the mechanisms by which endocannabinoids transport across cell membranes (Yates and Barker, 2009).

Over the last few years there have been several hypotheses proposed for the transport mechanism of AEA. These have included a passive diffusion model (Fasia et al., 2003, Glaser et al., 2003, Kaczocha et al., 2006) and a protein-facilitated uptake process, the nature of which up until recently remained elusive. Fu et al., describe a cytosolic variant of the AEA-degrading enzyme FAAH, FAAH-like AEA transporter (FLAT). FLAT has the ability to bind AEA and facilitate its translocation across membranes into cells (Fu et al., 2012). Evidence of this comes from AEA transport inhibitors, AM404 and OMDM-1, which block the effects of FLAT. A competitive antagonist of the FLAT-AEA interaction, a phthalazine derivative ARN272, prevents AEA internalization *in vitro* and interrupts its deactivation *in vivo*. The overall effect of FLAT is to increase AEA levels in the cell. Fatty acid binding proteins (FABPs) are also involved in AEA transport and can deliver AEA across the cytosol to the degrading enzyme FAAH (Kaczocha et al., 2009).

In contrast, Fu et al., found that FLAT expression did not contribute to 2-AG transport across cells nor did it increase 2-AG levels (Fu et al., 2012). Relatively little is known about the uptake mechanism for 2-AG, however it is important to note that while AEA is taken up into postsynaptic neurons 2-AG is transported presynaptically into the neuron (Di Marzo, 2008, Di Marzo et al., 2004, Di Marzo et al., 2015) indicating the two may have separate transporter mechanisms.

1.4.9 Endocannabinoid degradation

Following its cellular uptake, AEA is primarily degraded to arachidonic acid and ethanolamine by the enzyme FAAH, located in the endoplasmic reticulum of the postsynaptic neuron (Cravatt et al., 1996, Otrubova et al., 2011, Dinh et al., 2002). FAAH also has the ability to metabolize other substrates including 2-AG, PEA and OEA. These compete with AEA for binding to FAAH, resulting in an entourage effect (Cravatt et al., 2001). While FAAH does have some ability to catabolise 2-AG, 2-AG is primarily metabolized to arachidonic acid and glycerol by the enzyme monoacylglycerol lipase (MAGL) (Ueda et al., 2011). Although FAAH and MAGL are the best characterised degrading enzymes (see Figure 1.5), both AEA and 2-AG can be metabolised by cyclooxygenase type 2 into prostaglandin ethanolamide and prostaglandin glycerol ester respectively (Kozak et al., 2002, Yu et al., 1997). While MAGL degradation accounts for most of the 2-AG metabolism in the brain, both ABHD6 and ABHD12 also contribute to 2-AG hydrolysis (Savinainen et al., 2012).

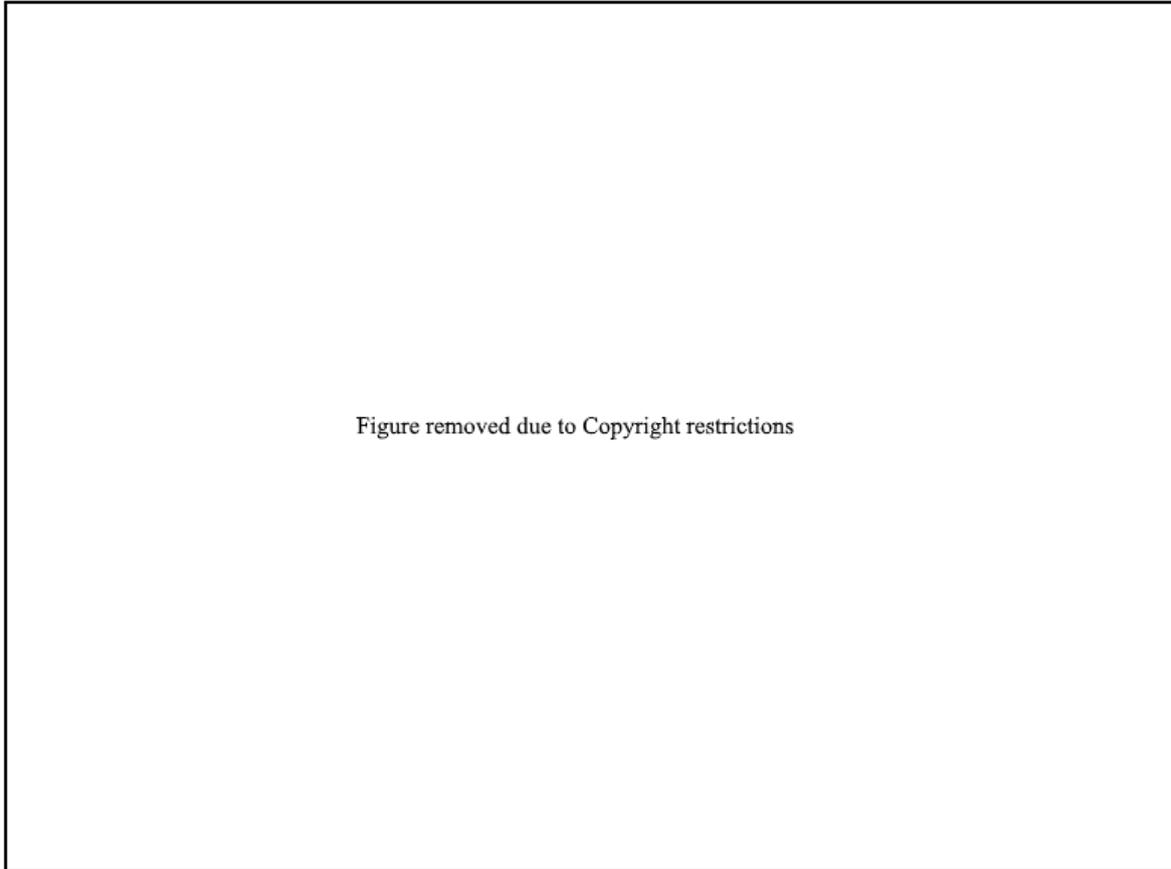


Figure 1.5: Schematic representation of endocannabinoid synthesis and degradation. 2-AG biosynthesis is mostly localised to the plasma membrane of postsynaptic neurons. AEA biosynthesis also occurs in the postsynaptic neuron. FAAH degradation of AEA is also found in the postsynaptic neuron while 2-AG degradation via MAGL occurs in the presynaptic terminal. 2-AG, 2-arachidonyl glycerol; AEA, AEA; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; (Di Marzo et al., 2004).

1.5 The endocannabinoid system and pain

The components of the endocannabinoid system are expressed throughout the pain pathways and regulate nociceptive processing at a number of different levels including the periphery (Burston and Woodhams, 2014, Woodhams et al., 2017), the spinal cord (Burston and Woodhams, 2014, Woodhams et al., 2017) and in supraspinal regions of the brain (discussed in more detail later), (Corcoran et al., 2015). In preclinical studies, increased endocannabinoid signalling has been found to elicit antinociceptive effects in animal models of acute pain, and chronic inflammatory and neuropathic pain (Herzberg et al., 1997, Seyrek et al., 2010, Zhu et al., 2009, Tsou et al., 1998a).

1.5.1 Peripheral

Mounting evidence has demonstrated that the endocannabinoid system plays a substantial role in peripherally mediated antinociception. Both neuronal and non-neuronal cannabinoids contribute to the analgesic effects of the periphery. Peripheral nociceptor CB₁ knockout (KO) mice have reduced analgesia produced by both local and systemic, but not intrathecal, cannabinoid agonists (Agarwal et al., 2007). Peripherally administered AEA and PEA, both alone and synergistically, attenuate formalin-evoked nociceptive behaviour (Calignano et al., 1998). Intra-plantar 2-AG or the MAGL inhibitor URB602 produced dose-dependent decreases in formalin-evoked nociceptive behaviour in rats, effects attenuated by CB₂ but not CB₁ antagonism (Guindon et al., 2007). Intra-plantar administration of a CB₁ or CB₂ agonist (ACEA or AM1241) was antinociceptive in the rat model carrageenan inflammation, effects blocked by CB₁ or CB₂ antagonism respectively (Gutierrez et al., 2007).

Intra-plantar URB597 attenuated hyperalgesia in the rat carrageenan model of inflammatory pain in the hind paw (Jhaveri et al., 2008b, Sagar et al., 2008), and increased levels of AEA, 2-AG and PEA in the hind paw (Jhaveri et al., 2008b). The MAGL inhibitor JZL184, injected intra-plantar to rats, suppressed capsaicin-evoked thermal hyperalgesia via CB₁ and CB₂ receptor mechanisms and capsaicin-evoked mechanical allodynia via a CB₁ receptor-dependent mechanism (Spradley et al., 2010). Dorsal paw injection of JZL184 produced antinociceptive effects in both phases of the rat formalin test, increasing 2-AG levels, via peripheral CB₁ and CB₂ receptors (Guindon et al., 2011). Subcutaneous, intraperitoneal or oral administration of the peripherally restricted FAAH enzyme inhibitor URB937 has been shown to attenuate nociception in rat and mice models of peripheral nerve injury and inflammatory

nociception via a CB₁ receptor-dependent mechanism (Clapper et al., 2010, Sasso et al., 2015). In summary, increasing the availability or action of peripheral cannabinoids produces largely antinociceptive effects via CB₁ or CB₂ receptors. Targeting these mechanisms of analgesia may have therapeutic benefits in treating pain disorders.

1.5.2 Spinal

At the level of the spinal cord, direct application of cannabinoids is antinociceptive in a rat model of thermal pain (Hohmann et al., 1999, Hohmann et al., 1998). Exogenous application of AEA intrathecally is antinociceptive in the chronic constriction injury (CCI) model of neuropathic pain in rats or mice (Welch et al., 1998, Starowicz et al., 2012). CB₁ receptor antagonism in the spinal cord can increase nociceptive firing of neurons in the dorsal horn leading to hyperalgesia in mice and rats (Richardson et al., 1997, Chapman, 1999). Intrathecal administration of CP55940 or the CB₂ agonist JW015 attenuated postoperative hypersensitivity in the rat paw incision surgery model (Romero-Sandoval and Eisenach, 2007). AEA and 2-AG may serve functionally distinct roles in the surgical incision model of acute resolving pain in rats. AEA levels are reduced in the spinal cord at time points associated with maximal mechanical hypersensitivity and return to baseline levels as the nociceptive behaviour subsides. 2-AG on the other hand is elevated during this time. These effects are associated with downregulation of CB₁ and upregulation of CB₂ receptors (Alkaitis et al., 2010).

In a rat model of spinal nerve ligation, intrathecal administration the FAAH inhibitor URB597 reduced mechanically evoked responses in spinal neurons of sham-operated and spinal nerve ligated rats (Jhaveri et al., 2006). Intrathecal administration of the MAGL inhibitor JZL184 dose-dependently reduced mechanically evoked nociceptive transmission in the spinal cord of naïve rats, an effect blocked by the CB₁ receptor antagonist AM251. JZL184 also abolished inflammation-induced expansion of the receptive field of spinal neurons in an intra-plantar carrageenan model of inflammation (Woodhams et al., 2012). Intrathecal AM251 decreased capsaicin induced hyperalgesia in mice while URB597 or the endocannabinoid re-uptake inhibitor UCM707 prolonged hyperalgesia. Furthermore, CB₁ KO mice but not mice deficient of CB₁ only in the primary afferent nociceptors did not develop capsaicin-induced hyperalgesia. Moreover, intrathecal injection of the CB₁/CB₂ agonist CP55940 increased hyperalgesia in wild type and CB₁ deficient mice but not CB₁ KO mice (Pernia-Andrade et al., 2009). Diminished synaptic inhibition in the dorsal horn of the spinal cord is a major

contributor to chronic pain (Sivilotti and Woolf, 1994, Yaksh, 1989, Treede and Magerl, 2000). In vitro electrical field stimulation of the superficial dorsal horn suggest that upon strong nociceptive stimulation CB₁ receptors exert their effects by reducing synaptic release of inhibitory GABA and glycine (Pernia-Andrade et al., 2009), accounting for the *in vivo* results presented in this study.

Furthermore, CB₁ receptor expression is upregulated in the spinal cord in the rat CCI model of neuropathic pain. This upregulation of CB₁ enhanced the antinociceptive effect of an intrathecal CB₁ agonist on both thermal hyperalgesia and mechanical allodynia (Lim et al., 2003). CB₂ receptor expression is also upregulated in the lumbar spinal cord in a rat model of neuropathic pain (Zhang et al., 2003). CB₂ KO mice expressed enhanced contralateral mechanical and thermal allodynia following sciatic nerve ligation, an effect protected against following overexpression of CB₂ receptors (Racz et al., 2008). The spinal cord represents an important region for analgesic activity in acute and chronic pain. Enhancing endocannabinoid signalling at the level of the spinal cord could have therapeutic implications for treating multiple pain disorders.

1.5.3 Supraspinal

Considerable effort has been invested in investigating the brain regions involved in mediating the antinociceptive effects of endocannabinoids and cannabinoid receptor agonists. Later sections will discuss in more detail the role of the endocannabinoid system in individual brain regions (RVM, PAG, amygdala and PFC) in pain and its modulation by stress. Presented in this section is an overview of the studies that have identified a role of the endocannabinoid system, supraspinally, in the modulation of pain.

The supraspinal sites of endocannabinoid mediated antinociception were first identified via microinjection of the cannabinoid agonist WIN 55,212-2 into brain regions associated with pain processing including the RVM and PAG prior to tail flick tests in rats (Martin et al., 1995). Strong evidence of a role for the supraspinal endocannabinoid system in the modulation of pain was provided by Hohmann et al., (1999) who demonstrated that systemic administration of WIN55,212-2 resulted in an antinociceptive effect in the tail flick test in rats. Transection of the spinal cord, and thus blockade of descending pain processes, inhibited the cannabinoid-induced suppression of noxious heat-evoked activity in the tail flick test, thus indicating that WIN55,212-2 acted supraspinally to mediate its antinociceptive efficacy. These studies paved

the way for the investigation of the role of supraspinal sites in cannabinoid-induced antinociception.

The antinociceptive activity of cannabinoid receptor agonists has been demonstrated in the mouse and rat tail flick tests following intracerebroventricular (i.c.v.) administration. Specifically, i.c.v. injection of the cannabinoid receptor agonists WIN55,212-2, THC and CP-55,940 produced antinociception in the rat tail flick test (Lichtman et al., 1996, Martin et al., 1993). In spinally transected rats, i.c.v. administration of the CB₁ receptor antagonist/inverse agonist rimonabant completely blocked the antinociceptive effects of systemically administered THC and CP 55,940 in the rat tail flick test, indicating that these effects are mediated through CB₁ receptors in the brain. The antagonist failed to block the effects of morphine, indicating its selectivity for CB receptors (Lichtman and Martin, 1997). However, when administered i.c.v., THC enhances the antinociceptive potency of morphine (Welch et al., 1995) suggesting a synergistic interaction between the opioid and cannabinoid systems. Similar to Martin et al., 1993, i.c.v. injection of WIN 55,212-2 and THC produced dose-related antinociceptive effects in the mouse tail flick test (Raffa et al., 1999). In addition, i.c.v. administration of WIN 55,212-2 induces antinociception in the mouse tail flick and paw withdrawal test (Fang et al., 2012). Thus, these results indicate that supraspinal CB₁ receptors are important in modulating pain processing.

Systemic WIN 55,212-2 dose-dependently inhibited stimulus-evoked activity, in the form of graded pressure stimuli to the paw, of nociceptive neurons in the ventroposterolateral thalamus (VPL) of anesthetized rats (Martin et al., 1996). Further evidence for a role of CB₁ receptors in the thalamus in mediating and modulating nociceptive responding came from work demonstrating that microinjection of WIN 55,212-2 into the thalamus resulted in antinociceptive effects in the tail flick test in rats (Martin et al., 1999). Similar effects were observed following microinjection into amygdala, superior colliculus and the noradrenergic A5 region (Martin et al., 1999). Furthermore, intra-locus coeruleus microinjection of the hypothalamic peptide orexin-A decreased formalin-evoked nociceptive behaviour in rats (Mohammad-Pour Kargar et al., 2015), an effect reversed by pre-treatment with either the OX1 receptor antagonist SB-334867 or the CB₁ receptor antagonist AM251. Intra-locus coeruleus microinjection of SB-334867 and AM251 alone induced hyperalgesia. The results from this study suggest a new mechanism by which orexin-A modulates nociceptive information in the locus coeruleus via interaction with CB₁ receptors.

There is now increasing evidence supporting the role of supraspinal CB₂ receptors in the modulation of pain (for review see Guindon & Hohmann (2008)). For example, microinjection of the CB₂ receptor agonist JWH-133 into the VPL of the thalamus has been shown to reduce spontaneous noxious mechanically evoked VPL neuron responses, in a rat model of neuropathic pain (spinal nerve ligation; SNL), an effect blocked by CB₂ antagonism via SR144528 (Jhaveri et al., 2008a).

The endocannabinoid system has also been proposed to play a role migraine-related pain (for review see Greco et al., (2010), Russo (2004) and Smith & Wagner, (2014)). FAAH deficient mice (FAAH (-/-)) express less nitroglycerin-induced migraine-like pain, with similar effects observed following pharmacological inhibition of FAAH inhibitors using URB597 and PF3945. Administration of the CB₁ receptor antagonist rimonabant blocked these antinociceptive effects in this migraine model, demonstrating a key role for CB₁ receptors in mediating the effects of the FAAH substrates (i.e. AEA) (Nozaki et al., 2015). Similarly, several other studies have demonstrated that genetic and/or pharmacological inhibition of FAAH is associated with increased AEA levels in the brain, and associated with antinociceptive effects in several pain models (Kwilasz et al., 2014b, Lichtman et al., 2004b). For example, URB597 (intra-peritoneal), the FAAH inhibitor, produced antinociception in the form of CB₁ dependant decreases in acid-stimulated stretching in a lactic acid model of pain, an effect was associated with increased AEA levels in the brain (Kwilasz et al., 2014b). Increased FAAH activity and an increased density of cannabinoid binding sites have also been found in the hypothalamus in animal models of migraine (nitroglycerin-induced hyperalgesia), (Greco et al., 2010b). It is clear that elevation of brain endocannabinoid levels produces robust antinociceptive effects in mouse models of migraine-related pain (Cravatt et al., 2001, Holt et al., 2005, Jayamanne et al., 2006, Lichtman et al., 2004a, Lichtman et al., 2004b).

As previously mentioned, endocannabinoids act on other non-CB₁/non-CB₂ receptors, such as the ligand-gated ion channel, TRPV1. TRPV1 on primary afferent neurons plays a key role in the sensation of pain and thermal hyperalgesia (Caterina et al., 2000). However increasing evidence suggests a role for TRPV1 in in supraspinal regions in pain modulation (Madasu et al., 2015). I.c.v. administration of the TRPV1 antagonist A-784168 reduced both weight bearing in a rat model of osteoarthritis and chronic inflammatory thermal hyperalgesia following administration of CFA to rats (Cui et al., 2006). Moreover, i.c.v. administration of TRPV1 antagonists reduced nociceptive behaviour in the rat formalin test (Santos and Calixto, 1997). Following spinal cord injury, CB₁ and TRPV1 receptors interact and play a role in the

plastic changes that occur in the rat brain (Knerlich-Lukoschus et al., 2011). In the same study, seven days following spinal cord lesion, CB₁ receptor immunoreactivity was induced in the thalamus and hippocampus and downregulated in the ACC, amygdala and PAG, brain regions related to pain, emotion, learning, and memory in rats. Double labelling found that TRPV1 was co-expressed with CB₁. Thus, alterations in CB₁-TRPV1 expression/activity may underlie the emotional-affective and somatosensory pain responses following spinal cord lesion.

Paracetamol (acetaminophen) is a well-recognised and potent analgesic drug (Toms et al., 2008) and a number of recent studies have demonstrated that paracetamol is metabolized by FAAH to form the TRPV1 agonist/AEA reuptake inhibitor AM404, which contributes to the antinociceptive activity of paracetamol (Hogestatt et al., 2005, Mallet et al., 2010, Zygmunt et al., 2000). The breakdown of paracetamol to AM404 occurs in the brain and is dependent on FAAH (Hogestatt et al., 2005). The de-acetylated paracetamol metabolite 4-aminophenol and 4-hydroxy-3-methoxybenzylamine (HMBA), produces antinociception in a variety of rodent (both mice and rats) pain tests (Barriere et al., 2013) and is metabolised in the brain to form AM404 plus HPODA or arvanil plus olvanil. The antinociceptive effects of arvanil were dependent on FAAH, TRPV1 and CB₁ receptors. FAAH-dependent generation of TRPV1-active analgesic drug metabolites may be useful in the production of novel pain therapeutics (Barriere et al., 2013).

GPR55, a putative novel cannabinoid receptor, has recently been shown to be involved in the development of hyperalgesia in models of inflammatory and neuropathic pain. Inflammatory mechanical hyperalgesia was absent in GPR55(-/-) knockout mice (Castane et al., 2006, Staton et al., 2008). Furthermore, following partial sciatic nerve ligation, GPR55(-/-) mice failed to express mechanical hyperalgesia (Staton et al., 2008). Together, these results suggest a pronociceptive role for GPR55, although only one study to date has investigated the role of this novel target supraspinally in the modulation of pain or stress (Deliu et al., 2015).

The PPARs are also targets for endocannabinoids and may play a role in endocannabinoid-induced analgesia. PPAR γ agonists produced anti-inflammatory and anti-hyperalgesic effects in carrageenan-treated rats, effects which were supra-spinally mediated (Morgenweck et al., 2010). Similarly, i.c.v. administration of PPAR α ligands produced anti-inflammatory and anti-hyperalgesic effects in mice and rats in the carrageenan model of inflammation, (D'Agostino et al., 2009, D'Agostino et al., 2007, Taylor et al., 2005). Thus, central PPARs play an important role in inflammatory nociceptive processing and responding.

Although i.c.v. administration of pharmacological agents is a useful means of investigating the contribution of the brain in general, alternative approaches are required to study the role of the endocannabinoid system in specific brain regions in pain. These approaches and the results obtained are discussed later for each of the key brain regions that comprise the descending pain pathways (RVM, PAG, amygdala and PFC). See Figure 1.6 for a synthesis of the literature reviewed herein on the role of the supraspinal endocannabinoid system in discrete brain regions in pain.

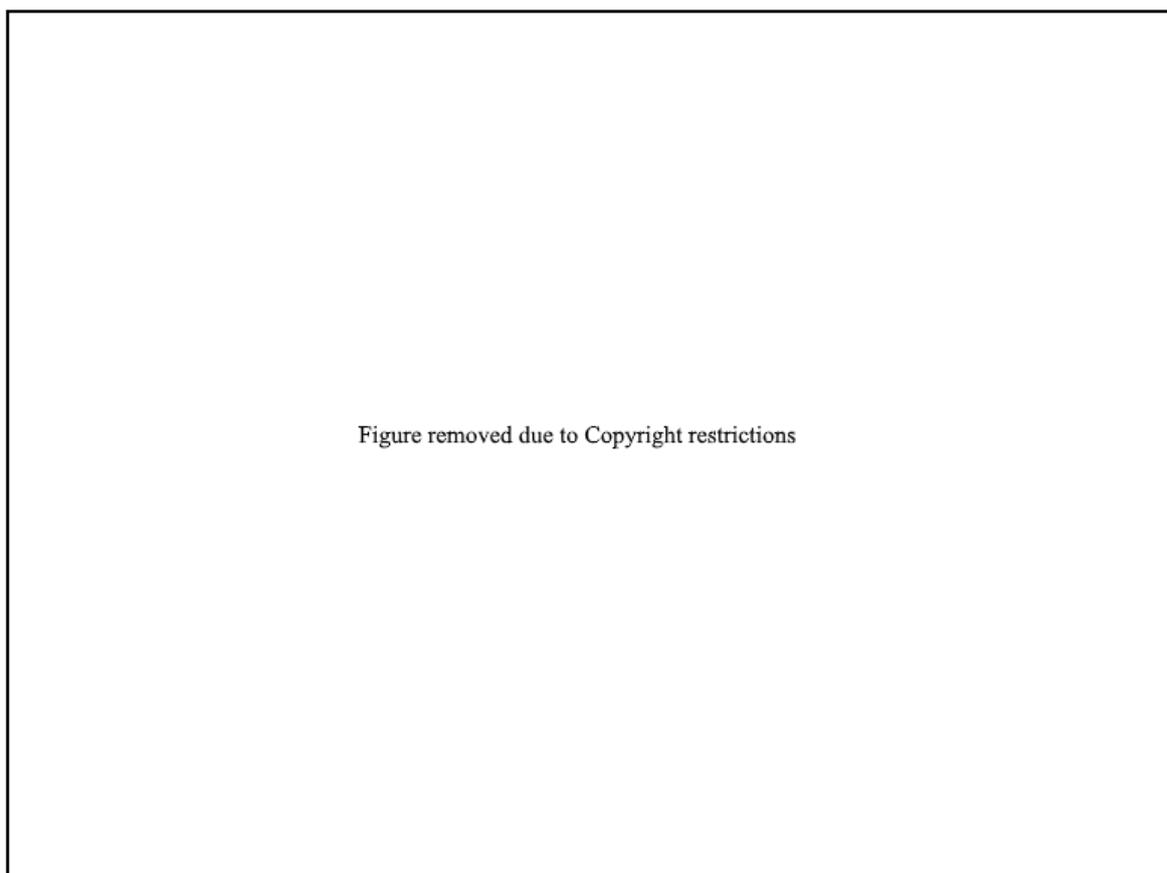


Figure 1.6: A synthesis of the literature reviewed herein on the role of the supraspinal endocannabinoid system in discrete brain regions in pain. mPFC: medial prefrontal cortex; ACC: anterior cingulate cortex; PrL: prelimbic cortex; IL: infralimbic cortex; BLA: basolateral amygdala; CeA: central nucleus of the amygdala; PAG: periaqueductal grey; RVM: rostral ventromedial medulla; GiA: gigantocellular reticular nucleus; TRPV1: transient receptor potential vanilloid 1; PPARs: peroxisome proliferator-activated receptors.

1.6 The endocannabinoid system in the brain regulates fear/anxiety/stress

1.6.1 Anxiety

The endocannabinoid system is distributed in brain regions and neuronal circuits associated with anxiety (Ruehle et al., 2012, Tovote et al., 2015, Mechoulam and Parker, 2013). Several studies using pharmacological and genetic approaches have indicated a role of the endocannabinoid system in anxiety behaviour (Lutz et al., 2015).

Transgenic studies involving FAAH and CB₁ receptor KO mice have revealed several important findings regarding the role of the endocannabinoid system in anxiety modulation. CB₁ receptor KO mice show decreased exploration in the open arms of the elevated plus maze compared to wild type controls, indicating an anxiogenic phenotype (Haller et al., 2002, Haller et al., 2004, Uriguen et al., 2004, Jacob et al., 2009). CB₁ receptor KO mice also show increased anxiety-like behaviour in the open field and light/dark test (Maccarrone et al., 2002, Uriguen et al., 2004, Martin et al., 2002, Jacob et al., 2009), as well as the social interaction test (Uriguen et al., 2004). In contrast to this anxiogenic phenotype, CB₁ receptor KO mice exposed to the marble shock probe burying test showed decreased burying behaviour and fewer contacts with the shock probe, indicating an anxiolytic phenotype (Degroot and Nomikos, 2004). Similarly an anxiolytic phenotype has been exhibited in FAAH KO mice in the light/dark and elevated plus maze tests (Moreira et al., 2008). In contrast, another study has shown that FAAH KO mice fail to exhibit any changes in anxiety-like behaviour in the elevated plus maze (Naidu et al., 2007). The differences in behaviour seen in these KO mice may be due to the different methodological approaches undertaken between the studies (i.e. open field vs. marble shock probe burying test). It is possible that while CB₁ receptors are anxiolytic in one test, they may be anxiogenic in another. In addition, it is possible that CB₁ receptor KO leads to a compensatory activation of TRPV1 leading to increased anxiety-like behaviour in certain paradigms (Rubino et al., 2008b). Moreover, FAAH KO may lead to an increase in AEA which is then available to act at either CB₁ and TRPV1, receptors known to have opposing effects in anxiety-related behaviour, which may account for the between-study differences seen. Humans and mice with a genetic polymorphism in FAAH (C385A: rs324420) leading to destabilised FAAH enzyme activity exhibit an anxiolytic phenotype in a self-report test (humans) and elevated plus test (mice) respectively (Dincheva et al., 2015). Despite these contrasting results the endocannabinoid system plays a key role in modulating anxiety-like behaviour. More targeted KO studies in discreet brain regions may be able to further our knowledge of the endocannabinoid system in anxiety-related phenotypes.

Pharmacological blockade of CB₁ receptors with rimonabant increased anxiety-like behaviours in the elevated plus maze and defensive withdrawal test in rats (Arevalo et al., 2001, Navarro et al., 1997). Interestingly the cannabinoid receptor agonist CP 55,940 also had an anxiogenic effect in the rat elevated plus maze (Arevalo et al., 2001). CP 55,940 is a non-selective cannabinoid receptor agonist. It is possible that it is therefore exerting its effects at a receptor other than CB₁ which would explain why both an antagonist and agonist had similar effects in this study. In contrast to the above, rimonabant decreased anxiety in wild type and CB₁ receptor KO mice in the elevated plus maze test (Haller et al., 2002, Rodgers et al., 2003), which may indicate that the anxiolytic effect may be mediated by a receptor other than CB₁. Furthermore, the CB₁ receptor antagonist AM251 dose-dependently induced an anxiogenic phenotype in the elevated plus maze in wild type mice (Haller et al., 2004, Patel and Hillard, 2006, Rodgers et al., 2005) and rats (Sink et al., 2010b) but not CB₁ KO mice (Haller et al., 2004). CP 55,940 and WIN-55,212-2 induced an anxiolytic effect in the elevated plus maze (Patel and Hillard, 2006, Haller et al., 2004) an effect abolished by AM251 (Haller et al., 2004). AM251 also significantly increased c-Fos expression in the rat amygdala and nucleus accumbens following elevated plus maze testing (Sink et al., 2010b). In a clinical setting, rimonabant has also been shown to induce adverse anxiogenic effects in human diabetic and obese patients (Rosenstock et al., 2008, Scheen, 2008). More recently, systemic administration of the CB₁ agonist WIN 55,212-2, daily for 6 consecutive days before exposure to repeated social defeat stress, reduced anxiety-like behaviour in rats exposed to the elevated plus maze and decreased the accumulation of circulating inflammatory monocytes (Lisboa et al., 2018)

The cannabinoid receptor agonist THC can dose dependently produce an anxiolytic or anxiogenic effect in rats. Low doses injected directly into the PFC or ventral hippocampus produce anxiolytic effects in the rat elevated plus maze test while high doses of THC induce anxiogenic effects. In the BLA, low dose THC has the opposite effect to the PFC and ventral hippocampus producing anxiogenic effects, while high doses have no effect (Rubino et al., 2008a). This dose dependent effect has also been observed upon administration of other CB₁ receptor agonists. Low doses of nabilone, cannabidiol (rats) (Onaivi et al., 1990), CP 55,940 (rats) (Marco et al., 2004) and THC (mice) (Berrendero and Maldonado, 2002) produced anxiolytic effects in the elevated plus and light-dark box test respectively. High doses of nabilone (Onaivi et al., 1990), CP 55,940 (Marco et al., 2004), and HU-210 (Rodriguez de Fonseca et al., 1996, Giuliani et al., 2000) produced anxiogenic effects in the elevated plus and

defensive withdrawal behaviour tests in rats. These studies indicate the importance of both CB₁ and non CB₁ targets in regulation of anxiety by the endocannabinoid system.

FAAH inhibition by either systemic URB597 or URB532 produced anxiolytic effects in the elevated plus, open field, and the isolation-induced ultrasonic emission tests respectively (Kathuria et al., 2003, Hill et al., 2007, Rubino et al., 2008a), an effect attenuated by rimonabant (Kathuria et al., 2003) in rats. Similarly, to FAAH inhibition, intra-dorsolateral PAG administration of AEA or the CB₁ receptor agonist ACEA produced anxiolytic effects in the elevated plus maze in rats. The AEA transport inhibitor AM404 potentiated the effect of AEA while AM251 attenuated it (Moreira et al., 2007). AM404 has also been shown to produce anxiolytic effects in multiple other studies. Systemic administration of AM404 has been shown to produce dose-dependent anxiolysis in rats in the elevated plus, defensive withdrawal and separation-induced ultrasonic vocalisation tests (Bortolato et al., 2006). AM404 increased the levels of AEA but not 2-AG in the PFC and its effect was attenuated by rimonabant administration (Bortolato et al., 2006). The increase in the endogenous tone of AEA by FAAH inhibition tends to produce an anxiolytic effect. However, similar to CB₁ receptor agonist administration, low doses of AEA or the AEA analog methanandamide may be anxiolytic while high doses are anxiogenic in the two compartment black and white box test (mice) (Akinshola et al., 1999), elevated plus (rats) (Rubino et al., 2008b) and the light/dark box (rats) (Scherma et al., 2008) tests.

2-AG signalling has also been heavily implicated in the modulation of anxiety behaviours. DAGL α KO mice have reduced 2-AG brain levels and increased anxiety in the open field, light/dark box and novelty induced hypophagia tests. These anxiogenic effects were reversed upon administration of the MAGL inhibitor JZL184 (Shonesy et al., 2014, Jenniches et al., 2016). Accordingly, MAGL overexpression in the hippocampus of mice produced an anxiogenic phenotype in the open field and elevated plus maze tests (Guggenhuber et al., 2015). JZL184-induced MAGL inhibition produced anxiolytic effects in mice in the light/dark test (Lomazzo et al., 2015), elevated zero maze (Busquets-Garcia et al., 2011), elevated plus maze (Busquets-Garcia et al., 2011, Sciolino et al., 2011) and the marble burying assay (Kinsey et al., 2011b).

The effects of cannabinoid-based drugs on anxiety must be approached and interpreted cautiously. As can be seen from the above studies, the dose and nature of the drug employed may produce opposite effects leading to anxiogenic or anxiolytic effects. This may be due to

the various targets of both AEA and 2-AG. Cannabinoid receptors other than CB₁ have shown to produce modulating effects on anxiety. TRPV1 KO mice show reduced anxiety behaviour in the light/dark test and elevated plus maze compared to their wild type counterparts (Marsch et al., 2007). CB₂ receptors have also been implicated in the modulation of anxiety-like behaviours. Systemic administration of the CB₂ agonist β -caryophyllene produced anxiolytic effects in the elevated plus maze, open field and marble burying test in mice (Bahi et al., 2014). In addition, chronic but not acute systemic administration of the CB₂ receptor antagonist AM630 induced anxiolytic effects in the light dark box test and elevated plus maze test respectively in mice, this effect was blocked by pre-treatment with the CB₂ agonist JWH133 (Garcia-Gutierrez et al., 2012). Moreover, mice overexpressing CB₂ receptors exhibit reduced anxiety-like behaviours in the light dark box test and elevated plus maze test while CB₂ receptor KO in mice produced an anxiogenic response in these tests of anxiety (Garcia-Gutierrez and Manzanares, 2011, Ortega-Alvaro et al., 2011).

While it is clear that the endocannabinoid system plays a role in modulating anxiety-like behaviour, with the multitude of endocannabinoid drugs and targets further pharmacological and genetic experiments are warranted to fully elucidate the mechanisms by which the endocannabinoid system exerts its bimodal actions.

1.6.2 Fear:

The endocannabinoid system is distributed in brain regions and neuronal circuits associated with fear, and several studies employing pharmacological and genetic approaches have indicated a role of the endocannabinoid system in fear-related behaviour (Riebe et al., 2012, Ruehle et al., 2012, Akirav, 2011, Myers and Davis, 2006, Lutz et al., 2015).

CB₁-deficient mice have impaired long-term and within-session extinction behaviour in auditory fear-conditioning tests (Marsicano et al., 2002, Plendl and Wotjak, 2010, Kamprath et al., 2006). Only an intense footshock was able to induce a sustained fear response in mice lacking CB₁ receptors, showing the importance of the intensity of the stressor used when fear-conditioning (Kamprath et al., 2009). Mice lacking CB₁ receptors in cortical glutamatergic neurons have increased fear responses, while mice lacking CB₁ receptors in serotonergic neurons have decreased fear responses to cued fear recall tests (Dubreucq et al., 2012). This study revealed no effect for mice lacking CB₁ receptors in GABAergic neurons while another study using these mice revealed enhanced fear expression in auditory fear conditioning

(Llorente-Berzal et al., 2015). Deletion of CB₁ receptors from medial habenular neurons reduces fear-conditioned freezing and abolishes conditioned odour aversion in mice, an effect mediated by cholinergic transmission (Soria-Gomez et al., 2015). DAGL α KO mice have reduced 2-AG levels throughout the brain and reduced AEA levels in the amygdala, a region highly involved in fear-related behaviour (Jenniches et al., 2016). These mice also showed impaired extinction and increased fear-related freezing following a conditioned fear paradigm (Jenniches et al., 2016).

Treatment of mice with the CB₁ receptor antagonist rimonabant impaired short- and long-term, extinction behaviour in auditory fear-conditioning tests in a manner similar to CB₁ deficient mice (Marsicano et al., 2002, Plendl and Wotjak, 2010, Chhatwal et al., 2005). Rimonabant also prolonged freezing expression in mice exposed to cued fear recall tests (Dubreucq et al., 2012, Bowers and Ressler, 2015) and attenuated within-session extinction in fear-conditioned rats (Finn et al., 2004). Systemic administration of the CB₁ antagonist AM251 increased freezing during the conditioning phase of contextual fear-conditioning in mice. Intra-CeA AM251 impaired within-session extinction while intra-BLA AM251 impaired long-term extinction in an auditory fear-conditioning paradigm in mice (Kamprath et al., 2011). Similarly intra-IL AM251 impaired fear extinction in rats while intra-IL WIN 55,212-2 facilitated extinction in rats (Lin et al., 2009). Intra-IL AEA or AM404 attenuated fear-related responses in a contextual fear-conditioning paradigm in rats, an effect blocked upon AM251 pre-treatment (Lisboa et al., 2010). Interestingly AM251 seems to have opposite effects on fear expression in context or cued fear conditioning in rats. Arenos et al. showed reduced freezing to a context and increased freezing to a tone whereas Sink et al. showed the opposite effect upon AM251 treatment in rats (Arenos et al., 2006, Sink et al., 2010a). The CB₂ receptor antagonist AM630 decreased freezing behaviour in an auditory conditioned fear paradigm in mice while the MAGL inhibitor JZL184 enhances fear expression and impairs extinction in a CB₁ receptor-dependent manner (Llorente-Berzal et al., 2015).

Either systemic or i.c.v administration of the inhibitor of endocannabinoid breakdown AM404 dose dependently enhanced extinction in fear-conditioned mice, an effect blocked by rimonabant (Chhatwal et al., 2005, Pamplona et al., 2008, Bitencourt et al., 2008). Conditioned fear-related freezing expression was reduced in rats following intra-dorsolateral PAG AM404 or AEA, an affect abolished upon pre-treatment with AM251 (Resstel et al., 2008). Systemic administration of the FAAH inhibitor AM3506 before extinction training decreased fear-related behaviour in mice, an effect attenuated upon intra-amygdala rimonabant. Intra-BLA

AM3506 facilitated fear extinction in a CB₁ dependent manner (Gunduz-Cinar et al., 2013). Repeated social defeat over the course of 6 days prolonged fear expression and impaired fear extinction recall in mice, an effect reversed by WIN 55,212-2 (Lisboa et al., 2018).

These studies indicate that the endocannabinoid system plays a key role in innate and conditioned fear. In particular they highlight a role for the endocannabinoid system in the regulation of fear memory, and the expression and extinction of fear. Components of the endocannabinoid system are present in brain regions and neural circuits associated with fear, in particular the mPFC, hippocampus, amygdala, hypothalamus, and PAG (Tovote et al., 2015, Herkenham, 1991, Herkenham et al., 1991, Tsou et al., 1998a, Tsou et al., 1998b). Recent studies have begun to elucidate the underlying mechanisms of endocannabinoid modulation of fear responses. However, further pharmacological and genetic studies are required to fully understand the role of the endocannabinoid system in discrete brain regions with respect to the acquisition, consolidation, expression and extinction of fear-related behaviour. An increased understanding of these processes could offer new opportunities for more specific pharmacological approaches and new therapeutic strategies for the treatment of fear-related disorders.

1.6.3 Stress:

The endocannabinoid system plays an important modulatory role in both acute and chronic, physical and psychological stress (for review see Lutz et al., (2015), Riebe & Wotjak (2011), Ruehle et al., (2012) and Finn (2010)).

The endocannabinoid system can negatively regulate the neuroendocrine response to psychological stress (Gorzalka et al., 2008, Steiner and Wotjak, 2008). Acute restraint stress in rats has been shown to increase FAAH activity and decrease AEA tone in the amygdala (Hill et al., 2009). FAAH alterations are mediated by corticotropin releasing factor (Gray and Vecchiarelli, 2015). AEA decreases were associated with increased corticosterone concentrations in rats. These increased corticosterone levels were blocked by FAAH inhibition with URB597, an effect which in turn was blocked by AM251 co-administration into the BLA. The cannabinoid receptor agonist HU-210 decreased stress related responses while AM251 alone increased them (Hill et al., 2009). This finding suggests a clear role for the endocannabinoid system and the magnitude of the HPA response elicited. Glucocorticoids secreted in response to stress activate the HPA axis to suppress endocrine activation by

suppressing glutamate release and facilitating GABA release. In the hypothalamus, glucocorticoids induce an increase in both AEA and 2-AG to facilitate endocrine suppression in a CB₁ dependent manner in rats (Di et al., 2005, Evanson et al., 2010). In the PFC glucocorticoids induce an increase in 2-AG resulting in the inhibition of GABA release in a CB₁ dependent manner in rats (Hill et al., 2011). Acute restraint stress can also induce a switch from long-term depression to long-term potentiation in the bed nucleus of the stria terminalis after mPFC stimulation, an effect not seen in CB₁ KO mice, indicating a key role for the endocannabinoid system in this process (Glangetas et al., 2013).

Chronic stress also alters endocannabinoid signalling throughout the brain. CB₁ receptor signalling has been shown to be downregulated in several brain regions associated with emotional processing following chronic stress exposure (such as chronic unpredictable stress and chronic psycho-emotional stress) including the amygdala, nucleus accumbens, hippocampus, striatum, dorsal raphe nucleus and hypothalamus of rats and mice respectively (Hill et al., 2005, Rossi et al., 2008, Wang et al., 2010, Haj-Dahmane and Shen, 2014, Hill et al., 2013) .

Repeated stress involves exposure to a stressor repeatedly over time. The endocannabinoid system sensitizes over time during repeated stress and is involved in the habituation to the repeated stressor, thus decreasing the chances of chronic stress occurring (Patel et al., 2005, Patel et al., 2009). Repeated restraint stress increases concentrations of 2-AG while decreasing AEA concentrations and MAGL activity (Sumislawski et al., 2011, Patel et al., 2005, Hill et al., 2010, Rademacher et al., 2008). In the rat amygdala enhanced 2-AG may contribute to habituation in repeated stress while decreased AEA may contribute to increased glucocorticoid signalling (Hill et al., 2010).

The endocannabinoid system plays an important role in the stress response and is a key component of physiological coping mechanisms that are engaged to prevent the exacerbation of acute stress to chronic stress.

1.7 The role of the endocannabinoid system in the modulation of pain by fear, stress and anxiety

1.7.1 SIA/FCA:

To reiterate, SIA or FCA are forms of robust pain suppression that occurs during or following exposure to unconditioned (SIA) or conditioned (FCA) stress. Valverde et al., investigated the

interaction between cannabinoid receptors and opioid-mediated responses using the CB₁ deficient mice. Forced swimming in cold water (10°C) is reported to induce analgesia by a non-opioid NMDA-dependent mechanism while forced swimming in warm water (34°C) is reported to induce analgesia by an opioid-dependent mechanism. While CB₁ deficient mice exhibit normal responses to thermal, mechanical and visceral pain they have impaired expression of SIA in the hot plate test following forced swim at 34°C but not 10°C. As CB₁ receptor deficient mice exhibited impaired expression of opioid but not non-opioid-mediated SIA this study suggests that both the endocannabinoid and opioid systems may interact to mediate SIA (Valverde et al., 2000). Similarly, formalin-evoked nociceptive behaviour was decreased (i.e. FCA was expressed) and freezing increased upon re-exposure of rats to a conditioning chamber previously paired with footshock. Systemic administration of the CB₁ receptor antagonist rimonabant attenuated FCA in rats demonstrating a key role for CB₁ receptors in this form of conditional analgesia (Finn et al., 2004). Systemic administration of the FAAH inhibitor URB597 enhanced FCA in formalin treated rats. This enhancement was blocked following systemic administration of either a CB₁ (rimonabant) or CB₂ (SR144528) antagonist (Butler et al., 2008). Similarly, SIA in rats exposed to tail flick test following conditioned footshock tests was attenuated by systemic administration of the CB₁ receptor antagonists SR141617A or AM251 and enhanced following systemic administration of the FAAH inhibitor/TRPV1 antagonist AA-5-HT (Hohmann et al., 2005, Suplita et al., 2005, Kurrikoff et al., 2008). In the same SIA paradigm, intra-theal administration of either a FAAH or MAGL inhibitor, URB597 or URB602 respectively, enhances SIA in rats in a CB₁ receptor-dependent manner (Suplita et al., 2006). In the spinal cord, 2-AG plays a key role in endocannabinoid-mediated SIA in mice in the tail flick test via mGluR5 activation of a DAGL-2-AG-CB₁ pathway (Nyilas et al., 2009).

Studies have also demonstrated a role for specific brain regions in both FCA and SIA. These are important to help us understand the mechanisms by which this form of endogenous analgesia occurs. These approaches and the results obtained are discussed later for each of the key brain regions that comprise the descending pain pathway (RVM, PAG, amygdala and prefrontal cortex). While the studies in these sections have helped elucidate the role of the endocannabinoid system in supraspinal brain regions involved in SIA and FCA, there is still a paucity of research in this area. The lack of studies within each of these regions highlight a potential focus for future research. See Figure 1.7 for a synthesis of the literature reviewed

herein on the role of the supraspinal endocannabinoid system in discrete brain regions in SIA and SIH.

1.7.2 SIH:

SIH is an enhancement of nociception and exacerbation of pain following stress or anxiety. Unlike SIA/FCA, there is a paucity of studies investing the role of the endocannabinoid system in SIH. Rats exposed to daily partial restraint stress showed an enhanced visceromotor reflex to colorectal distention. Systemic injection of the CB₁ receptor agonist attenuated this effect while the CB₁ receptor antagonist rimonabant enhanced this effect. PCR and western blotting analysis also demonstrated an increase in CB₁ receptor expression in stressed rats (Shen et al., 2010). Similarly, stress-induced visceral hyperalgesia was increased in rats exposed to water avoidance stress daily for 10 days. AEA was increased in the dorsal root ganglia of stressed rats while CB₁ expression was decreased. Similarly, dorsal root ganglia treatment with AEA mimicked these receptor expression changes *in vitro*. Systemic administration of the CB₁ receptor agonist WIN55,212-2 prevented the development of stress-induced visceral hyperalgesia suggesting a role for CB₁ in this form of SIH (Hong et al., 2009). Chronic unpredictable stress (CUS) enhanced basal nociceptive responding, and, when paired with intramuscular nerve growth factor injections, induced a sustained and long-lasting hyperalgesia in mice. Both the FAAH and MAGL inhibitors, URB597 and JZL184, respectively, attenuated CUS-induced thermal hyperalgesia in mice in the hot plate test while increasing endocannabinoid levels in the brain and periphery. URB597 but not JZL184 attenuated the long-lasting hyperalgesia induced by the CUS and nerve growth factor combination indicating therapeutic potential of targeting the endocannabinoid system for the treatment of stress-induced exacerbation of pain (Lomazzo et al., 2015).

Studies have also demonstrated a role for specific brain regions in SIH which are important as they help us to understand the mechanisms by which this hyperalgesia occurs. These approaches and the results obtained are discussed later for each of the key brain regions that comprise the descending pain pathway (RVM, PAG, amygdala and prefrontal cortex). It is clear however that there is a scarcity of research investigating the role of the endocannabinoid system in SIH in spinal, peripheral and supraspinal systems. The lack of studies within this area highlight a potential focus for future research. Understanding the mechanisms behind SIH may help to identify future therapeutic targets. See Figure 1.7 for a synthesis of the literature

reviewed herein on the role of the supraspinal endocannabinoid system in discrete brain regions in SIH and SIA.

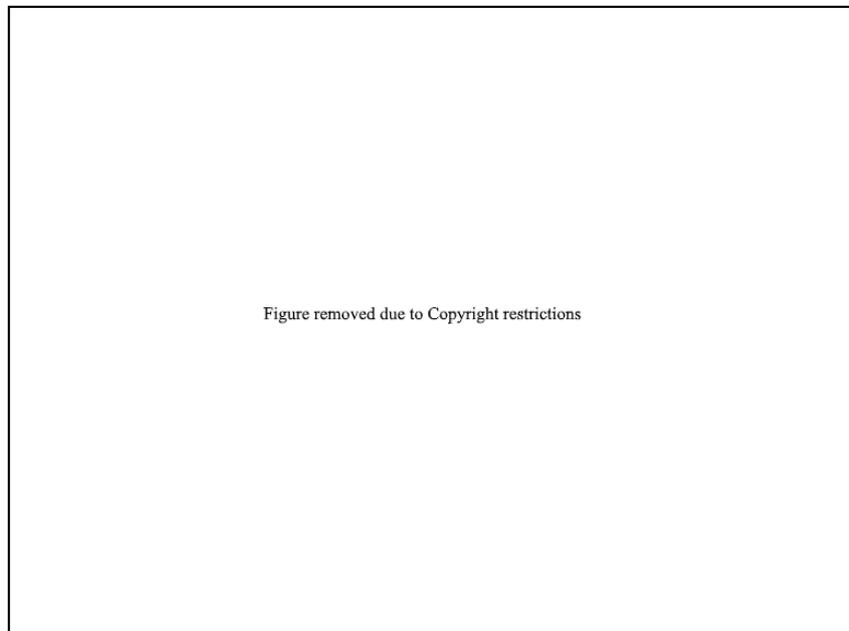


Figure 1.7: A synthesis of the literature reviewed herein on the role of the supraspinal endocannabinoid system in discrete brain regions in stress-induced analgesia (SIA) and stress-induced hyperalgesia (SIH). eCB: endocannabinoid ; BLA: basolateral amygdala; PAG: periaqueductal grey; dIPAG: dorsolateral PAG; RVM: rostral ventromedial medulla; TRPV1: transient receptor potential vanilloid 1; WKY: Wistar-Kyoto rat.

1.7.3 The role of the endocannabinoid system in the rostral ventromedial medulla (RVM) in pain, stress-induced analgesia and stress-induced hyperalgesia

1.7.3.1 Pain:

The RVM is made up of the nucleus raphe magnus, the nucleus gigantocellularis pars alpha (GiA) and the adjacent reticular formation; and is a major component of the descending pain pathway (Meng and Johansen, 2004). CB₁ receptors have been shown to be expressed in the RVM using receptor autoradiography and immunohistochemistry (Glass et al., 1997, Herkenham, 1991, Herkenham et al., 1991, Herzberg et al., 1997, Mailleux et al., 1992, Thomas et al., 1992, Tsou et al., 1998a). The RVM contains ON and OFF cells which are involved in descending facilitation and inhibition of nociception, respectively (Vanegas et al., 1984) and neuronal projections from the RVM to the dorsal horn of the spinal cord and the trigeminal

nucleus exert bi-directional control over nociception (Aicher et al., 2012, Basbaum and Fields, 1984).

The RVM shares connections with the PAG, forming the PAG-RVM pathway (Basbaum and Fields, 1984). Cannabinoids activate descending analgesia via this pathway through a process of ‘GABA disinhibition’. According to the GABA disinhibition hypothesis of analgesia, CB₁ receptor-mediated inhibition of GABAergic interneurons in the PAG and RVM results in disinhibition of projection neurons within the descending inhibitory pain pathway, resulting in analgesia (Basbaum and Fields, 1984, Lau and Vaughan, 2014, Szabo and Schlicker, 2005).

Microinjection of the CB receptor agonists WIN55,212-2 and HU210 into the RVM suppressed nociceptive behaviours in the tail flick test in rats, an effect attenuated by co-administration with the CB₁ receptor antagonist rimonabant (Martin et al., 1998). Nociceptive behaviour remained unchanged upon CB receptor agonist injection outside of the RVM. Cannabinoids induce antinociception by modulating neuronal activity in the RVM and inactivation of the RVM prevents CB-induced analgesia in rats (Meng and Johansen, 2004). As previously mentioned, ON-cells in the RVM increase firing in response to painful stimuli whereas OFF-cells decrease firing, facilitating and inhibiting pain respectively. Intra-RVM microinjection of WIN55,212-2 increases tail flick latencies while inhibiting ON-cell activity and increasing OFF-cell activity. Co-infusion with rimonabant blocked these effects, indicating a role for CB₁ receptors in the RVM in suppression of nociception (Meng and Johansen, 2004).

Microinjection of WIN 55,212-2 into the GiA resulted in behavioural analgesia in the rat tail flick test and the formalin test, an effect blocked by co-administration of rimonabant (Monhemius et al., 2001). In the same study, intra-GiA WIN 55,212-2 significantly decreased formalin-evoked nociceptive behaviour in partial sciatic nerve ligated rats, contralaterally to the site of nerve ligation, an effect reversed by intra-GiA rimonabant. This study demonstrated a role for the CB₁ receptor in GiA-mediated antinociception and modulation of nociceptive transmission in both acute pain and chronic neuropathic pain (Monhemius et al., 2001).

1.7.3.2 SIA/FCA:

Intra-RVM administration of rimonabant attenuated SIA in a rat model that combined footshock and a tail flick test. The FAAH inhibitor /TRPV1 antagonist AA-5-HT, administered systemically or intra-RVM, enhanced SIA in rats in a CB₁ receptor-dependent manner (Suplita

et al., 2005). This study provides evidence for an important role of CB₁ receptors in the RVM in mediating and modulating SIA.

1.7.3.3 SIH:

While there is evidence for a role of the RVM in SIH (for review see Jennings et al., (2014)), few studies have specifically investigated the role of the endocannabinoid system in the RVM in SIH. Genetic background plays a key role in determining the effect of stress on pain. As previously discussed, the WKY rat displays increased sensitivity to noxious stimuli and exhibits a depressive/anxiety-like phenotype and hyper-sensitivity to stress, compared with other rat strains including SD rats (Burke et al., 2010, O'Mahony et al., 2010). Our research group has recently reported an impairment in pain-related mobilization of the endocannabinoids AEA and 2-AG, along with their synthesising enzymes, NAPE-PLD and DAGL, respectively, in the RVM of WKY rats compared with SD rats, following intraplantar injection of formalin (Rea et al., 2014b). Systemic administration of AM251 potentiated while systemic administration of the FAAH inhibitor URB597 attenuated hyperalgesia to formalin injection in WKY rats, but not SD rats, an effect mediated by CB₁ receptors in the RVM. These data suggest that endocannabinoid dysfunction in the RVM underlies the hyper-sensitivity to noxious stimuli in the WKY rat model of negative affective state (Rea et al., 2014b).

1.7.4 The role of the endocannabinoid system in the periaqueductal grey (PAG) in pain, stress-induced analgesia and stress-induced hyperalgesia

1.7.4.1 Pain:

The PAG is a midbrain/brainstem structure that can be divided into four columns along its rostro-caudal axis: dorsomedial (dmPAG), dorsolateral (dlPAG), lateral (lPAG) and ventrolateral (vlPAG) columns (Bandler and Keay, 1996). Exposure to an aversive stimulus activates the descending inhibitory pain pathway, of which the PAG is a key component. The PAG, via its projections to the RVM, modulates nociceptive transmission at the level of the spinal cord (Fields et al., 1991). The PAG possesses a larger density of CB receptors than other brainstem structures (Herkenham et al., 1991). Cannabinoids act in the PAG to inhibit GABAergic and glutamatergic synaptic transmission and to produce analgesia by a disinhibitory mechanism (Vaughan et al., 2000).

CB₁ receptor-mediated antinociception and increased levels of AEA were reported following electrical stimulation of the dorsal PAG (dPAG) and IPAG (Walker et al., 1999). These authors also showed that subcutaneous injection of formalin elicited a pain response in rats and substantially increased AEA levels in the PAG, measured by *in vivo* microdialysis. Increased levels of the endocannabinoids, AEA and 2-AG, were also seen in the PAG and RVM of rats 7 days post CCI of the sciatic nerve, when hyperalgesia and mechanical allodynia were observed to be maximal (Petrosino et al., 2007). The authors propose that AEA and 2-AG are upregulated during CCI of the sciatic nerve possibly to decrease nociceptive behaviour.

Intra-vIPAG administration of morphine in rats enhanced the antinociceptive effect of the CB₁ receptor agonist HU-210 microinjected into the vIPAG in the hot plate test (Wilson-Poe et al., 2013). Likewise, intra-vIPAG and systemic administration of HU-210 in rats enhanced the antinociceptive effect of intra-vIPAG morphine (Wilson et al., 2008). Formalin-evoked nociceptive behaviour was reduced following microinjection of HU210 into the dPAG of rats, an effect blocked by co-administration with the CB₁ receptor antagonist rimonabant (Finn et al., 2003). These studies provide evidence for a dual role of opioid receptors and CB₁ receptors in pain and antinociception.

Microinjection of CP-55,940 into the posterior vIPAG, but not the posterior dIPAG or the anterior vIPAG, areas produces antinociception in the rat tail flick test (Lichtman et al., 1996). WIN55,212-2 increased tail-flick latencies following microinjection into the rat dIPAG, (Martin et al., 1995). Microinjection of WIN55,212-2 into the dIPAG increased the latency of the nociceptive response in the plantar test in rats, an effect blocked by co-administration with rimonabant. MPEP, a metabotropic glutamate receptor mGlu5 antagonist, also completely blocked the antinociceptive effect of WIN55,212-2 (Palazzo et al., 2001), indicating a CB₁-glutamatergic interaction in the PAG in mediating cannabinoid-induced analgesia.

Studies investigating the analgesic effects of nonsteroidal anti-inflammatory drugs (NSAIDs) in supraspinal structures indicate a role for endocannabinoids and CB₁ receptors in the PAG and RVM. Inflammation-induced hyperalgesia in rats can be attenuated by microinjection of the NSAID metaz inol into the PAG (Vazquez et al., 2007). Injection of the CB₁ receptor antagonist AM251 into the PAG or RVM reverses metaz inol-induced analgesia, suggesting a role for the endocannabinoid system in these brain regions in NSAID-induced analgesia in rats (Escobar et al., 2012).

TRPV1, a target of AEA, is expressed in the PAG (Palazzo et al., 2008) and a role for TRPV1 in pain modulation in the PAG has also been demonstrated. Intra-dIPAG injection of the TRPV1 agonist capsaicin increased the latency of nociceptive responses in the rat plantar test (Palazzo, et al., 2002). A higher dose administered to the same region produced opposite effects, decreasing the latency of nociceptive responses and inducing hyperalgesia followed by analgesia in rats (McGaraughty et al., 2003). Similar to Palazzo et al. (2002), intra-vIPAG administration of capsaicin also increased the latency of nociceptive responses in the hot-plate responses in rats (Liao et al., 2011) (for review see Starowicz et al., (2007)). Intra-dPAG administration of AEA is antinociceptive at TRPV1 receptors in a mouse tail flick model of pain following intra-dPAG pre-treatment with the CB₁ antagonist AM251 (Mascarenhas et al., 2017).

Our group has recently demonstrated a role for the TRPV1 in the PAG in genotype-dependant inflammatory nociception. Intra-dIPAG administration of the TRPV1 agonist or antagonist, capsaicin or 5'IRTX respectively, increased formalin-evoked nociceptive behaviour in SD but not WKY rats. Intra-vIPAG capsaicin was antinociceptive in SD rats and 5'IRTX was antinociceptive in both SD and WKY rats. Intra-lPAG 5'IRTX was antinociceptive in SD but not WKY rats (Madasu et al., 2016). Thus, TRPV1 modulation in the PAG elicits antinociceptive effects in several pain models in a genotype- and sub-column specific manner. For a recent review see Madasu et al., (2015) .

Intra-vIPAG injection of the FAAH inhibitor URB597 produced a robust hyperalgesic response at low doses, an analgesic response at high doses, and a biphasic effect on nociception at intermediate doses, in the rat plantar test (Maione et al., 2006). AEA and 2-AG levels were increased in a dose-dependent manner following URB597 administration into the vIPAG. Co-administration of a low dose of URB597 with the CB₁ receptor antagonist AM251 converted the hyperalgesic effect to an analgesic one, while co-administration of URB597 with both the TRPV1 antagonist capsazepine and AM251 abolished all effects. In comparison, the early hyperalgesic effect of the intermediate dose of URB597 was blocked by AM251, while the later URB597-induced analgesic effect became hyperalgesic following TRPV1 antagonism. CB₁ receptor-dependent analgesia was seen at the highest dose of intra-vIPAG URB597 administration (Maione et al., 2006). This study suggests a role for both CB₁ and TRPV1 receptors within the vIPAG in the endocannabinoid-mediated control of the descending pain pathway.

Diabetes is frequently associated with neuropathy, with many patients suffering from hyperalgesia or allodynia. A role for TRPV1 and CB₁ receptors in the PAG has been proposed in diabetic thermal hyperalgesia (Mohammadi-Farani et al., 2010). Intra-vIPAG administration of capsaicin and WIN55,212-2 produced antinociception in the hot plate test of non-diabetic mice (Mohammadi-Farani, et al., 2010). In contrast the antinociceptive effects of intra-vIPAG capsaicin and WIN55,212-2 were reduced in hyperalgesic diabetic mice, an effect associated with CB₁ receptor upregulation and TRPV1 downregulation in the vIPAG (Mohammadi-Farani et al., 2010). Taken together, the data demonstrate that diabetic neuropathy is associated with altered endocannabinoid signalling in the PAG, effects which may underlie the associated hyperalgesia and allodynia.

Systemic administration of the FAAH inhibitor/TRPV1 antagonist AA-5-HT produced antinociceptive effects in both rats and mice treated with formalin and in rats with CCI of the sciatic nerve (Maione et al., 2007), effects associated with increased levels of AEA in both the PAG and RVM. These antinociceptive effects were blocked by both CB₁ receptor and TRPV1 antagonists. Intra-vIPAG injection of AA-5-HT increased endocannabinoid levels and induced a pro-nociceptive effect at low doses and an antinociceptive effect at higher doses in the rat tail flick test (de Novellis et al., 2008). These effects were blocked by antagonism of vIPAG CB₁ receptors (AM251) or TRPV1 (I-RTX). Furthermore, administration of the FAAH inhibitor URB597 with the TRPV1 antagonist I-RTX into the vIPAG also induced antinociceptive effects in the rat tail flick test and inhibited RVM ON and OFF cell activity (de Novellis et al., 2008), thus indicating that the antinociceptive effects of FAAH substrates in the vIPAG may be mediated by CB₁ receptors. In the formalin test of inflammatory pain, intra-PAG AA-5-HT prevented the changes in the ON and OFF cell firing activity induced by intra-plantar injection of formalin. Since CB₁ and TRPV1 antagonists blocked the effects of AA-5-HT, (de Novellis et al., 2008), it suggests that these two endocannabinoid receptors in the PAG may be responsible for AA-5-HT-induced analgesia. Furthermore, Intra-PAG administration of the GPR55 agonist LPI reduced the nociceptive threshold in the rat hot plate test, an effect blocked upon pre-treatment with the GPR55 antagonist ml-193 (Deliu et al., 2015). This study suggests that altering GPR55 activity in the PAG may affect pain perception. Taken together these studies suggest that CB₁ receptors, TRPV1 and GPR55 in the PAG regulate pain behaviour. However further studies are required in order to determine the interaction between these three receptors in the modulation of this pain-related behaviour.

Orexin (OX) A and B are peptides and endogenous agonists for the OX1 and OX2 receptors which are localized in the lateral and perifornical area of the hypothalamus, (de Lecea et al., 1998, Sakurai et al., 1998, Tsujino and Sakurai, 2009). They exert antinociceptive effects (Chiou et al., 2010) following direct administration into the rat PAG (Azhdari Zarmehri et al., 2012). Orexin A decreases GABA release in an endocannabinoid-dependant manner in the vIPAG. Activation of OX receptors in the vIPAG leads to antinociception, measured electrophysiologically in rat brain slices. Intra-vIPAG microinjection of orexin A reduced hot-plate nociceptive responses in rats in a manner blocked by the CB₁ receptor antagonist/inverse agonist AM 251 (Ho et al., 2011).

1.7.4.2 SIA/FCA:

A number of studies have demonstrated an important role for the endocannabinoid system in the PAG in SIA/FCA. Intra-dPAG administration of the CB₁ receptor antagonist/inverse agonist rimonabant attenuated SIA, observed as an increase in the tail flick latency following exposure of rats to footshock stress (Hohmann et al., 2005). The same dose of this drug administered i.c.v., intra-vIPAG and intra-IPAG had no effect on SIA in this study, highlighting a particular role of CB₁ receptors in the dIPAG in mediating SIA. Increased levels of 2-AG were seen in the dIPAG directly after footshock stress, implicating this endocannabinoid in the dIPAG in SIA. Moreover, inhibition of the 2-AG degrading enzyme MAGL in the dIPAG using URB602 enhanced SIA (Hohmann et al., 2005). A subsequent study by the same group confirmed the CB₁ receptor-dependant attenuation of SIA following intra-dIPAG administration of rimonabant and the CB₁-dependant enhancement of SIA following AA-5-HT administration (Suplita et al., 2005). These studies provide evidence that the PAG is a key neural substrate for endocannabinoid-mediated SIA. Another follow-up study from this group showed that mGluR5 receptor activation mobilizes 2-AG in the dIPAG to produce SIA in rats (Gregg et al., 2012). Thus, unconditioned SIA mediated by CB₁ receptor stimulation in the PAG is under the control of glutamatergic neurotransmission via mGluR5 receptors.

Restraint stress can lead to SIA in the hot plate test in mice. Intra-vIPAG injection of the CB₁ receptor antagonist AM251 or OX1 antagonist SB334867 can attenuate this effect. The authors hypothesised that OXs are released into the vIPAG, activating OX1 receptors leading to endocannabinoid-mediated disinhibition and analgesia (Lee et al., 2016).

Our group has reported a role for the endocannabinoid system in the PAG in a model of SIA associated with conditioned, learned fear in (fear-conditioned analgesia; FCA) in rats (Olango et al., 2012). FCA in these studies was measured as the reduction of formalin-evoked nociceptive behaviour upon re-exposure of rats to a conditioning arena previously paired with footshock. Systemic administration of the FAAH inhibitor URB597 enhanced FCA, an effect associated with reduced phospho-ERK1/2 expression in the PAG (Butler et al., 2008). FCA was attenuated by intra-dIPAG administration of rimonabant (Olango et al., 2012), confirming a role for CB₁ receptors in the dIPAG in mediating both conditioned and unconditioned forms of SIA.

1.7.4.3 SIH:

While there is evidence for a role of the PAG in SIH (for review see Jennings et al., (2014)), there is currently a paucity of studies addressing the role of the endocannabinoid system in the PAG in SIH (with the exception of Madasu et al., (2016) which has already been discussed) and this is an area that warrants investigation.

1.7.5 The role of the endocannabinoid system in the amygdala in pain, stress-induced analgesia and stress-induced hyperalgesia

1.7.5.1 Pain:

The amygdala is a key region of the limbic system located in the medial temporal lobe. It contains many different nuclei including, the lateral nucleus (LA), BLA, CeA, accessory basal nucleus (ABA) and the medial nucleus (MeA). The amygdala plays a key role in the interaction between pain and emotion. The CeA, in particular, is involved in the emotional-affective component of persistent pain (Neugebauer et al., 2009, Neugebauer et al., 2004), while the BLA may be involved in the modulation of acute or tonic nociceptive processing (Oliveira & Prado, 1998). The amygdala is a key region of the ascending and descending pain pathways and shares connections with other key regions including the PFC and PAG. Pain-related changes have been identified in the amygdala in animals and humans using positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) studies (Neugebauer et al., 2004, Derbyshire et al., 1997, Becerra et al., 1999, Bingel et al., 2002, Bornhvd et al., 2002, Bonaz et al., 2002, Schneider et al., 2001, Petrovic et al., 1999, Paulson et al., 2002).

All components of the endocannabinoid system are expressed in the amygdala, although CB₁ receptors are expressed in highest density in the BLA (Herkenham, 1991, Tsou et al., 1998a). The amygdala contributes to the antinociceptive effects produced by systemically administered cannabinoids. WIN55,212-2 produces dose dependent antinociceptive effects in rats characterized as increased tail flick latencies (Manning et al., 2003). Intra-CeA, but not intra-BLA, administration of muscimol, significantly attenuated these antinociceptive effects of systemically administered WIN55,212-2. Moreover, unilateral CeA inactivation via muscimol reduced the suppression of formalin-evoked c-Fos expression by WIN55,212-2 in the superficial dorsal horn of the spinal cord but not in the deeper ‘nociceptive’ laminae (Manning et al., 2003). Another study from the same group found that the amygdala also plays a role in antinociception in non-human primates (Manning et al., 2001). WIN55,212-2 produced dose-dependent analgesia in rhesus monkeys. Bilateral lesions to the amygdala of the monkeys significantly reduced cannabinoid-induced analgesia. Both of these lesion studies indicate that the endocannabinoid system in the amygdala, in particular the CeA, can mediate antinociceptive effects.

Tail flick latencies have been shown to be increased upon microinjection of WIN55,212-2 into the CeA and BLA in rats (Ghalandari-Shamami et al., 2011, Hasanein et al., 2007, Martin et al., 1999). Furthermore, intra-BLA administration of WIN55,212-2 has also been shown to reduce formalin-evoked nociceptive behaviour in rats (Haghpourast et al., 2012, Hasanein et al., 2007), an effect attenuated by intra-BLA administration of the CB₁ receptor antagonist AM251 (Hasanein et al., 2007). Interestingly, intra-BLA administration of rimonabant has also been shown to attenuate formalin-evoked nociceptive behaviour and associated increases in c-Fos immunoreactivity in the hippocampus and RVM in rats (Roche et al., 2010, Roche et al., 2007) although intra-BLA administration of a different CB₁ receptor antagonist, AM251, did not exert a similar effect (Rea et al., 2013).

Using fMRI, it has been shown that the amygdala may play a role in the modulation of pain perception by Δ^9 -THC in humans (Lee et al., 2013). Cutaneous ongoing pain and hyperalgesia induced by capsaicin were monitored in healthy cannabis-naive volunteers. Δ^9 -THC reduced ‘painfulness’ but not the intensity of pain and hyperalgesia an effect positively correlated with amygdala activity. A Δ^9 -THC-related reduction in sensory-limbic functional connectivity was also seen between the amygdala and primary sensorimotor areas (Lee et al., 2013).

While the evidence points to a clear role for the endocannabinoid system in the amygdala in antinociception, there is a paucity of studies investigating its impact on the emotional aspect of pain. As a region with a clear role for the interaction between pain and emotion, it is necessary to further investigate this area and the role of the endocannabinoid system therein.

1.7.5.2 SIA/FCA:

The amygdala plays a role in both unconditioned and conditioned SIA (Helmstetter, 1992, Helmstetter and Bellgowan, 1993, Helmstetter et al., 1995, Werka, 1994, Werka, 1997, Werka and Marek, 1990). Intra-BLA microinjection of rimonabant has been shown to suppress SIA in rats exposed to footshock stress and then tested in the tail flick test, whereas intra-CeA microinjection had no effect on SIA (Connell et al., 2006). Intra-BLA administration of FAAH and MAGL inhibitors, however, had no effect on SIA (Connell et al., 2006), suggesting that CB₁ receptors in the BLA, but not CeA, mediate SIA, although the role of the individual endocannabinoids require further investigation. Roche et al. (2007 and 2010) reported no effect of unilateral or bilateral intra-BLA administration of rimonabant on FCA in rats (Roche et al., 2010, Roche et al., 2007). However, a subsequent study showed that the expression of FCA in rats was reduced following systemic or intra-BLA, but not intra-CeA, administration of a different CB₁ receptor antagonist, AM251 (Rea et al., 2013).

URB597 enhances the expression of FCA in rats when administered via the intra-peritoneal route, an effect blocked by either CB₁, CB₂ or μ -opioid receptor antagonists. Interestingly, FCA in this study was associated with increased expression of phospho-ERK2 in the amygdaloid complex. In contrast, the URB597-induced enhancement of FCA was associated with reduced phospho-ERK1 and phospho-ERK2 expression in the amygdala. This dichotomy is not consistent with a causal role of ERK signalling in the amygdala in FCA (Butler et al., 2008).

CB₁ receptors are expressed on GABAergic and glutamatergic neurons in the BLA (Herkenham, 1991, Katona et al., 2001). Expression of FCA in rats was reduced following systemic or intra-BLA, but not intra-CeA, administration of the CB₁ receptor antagonist/inverse agonist AM251 (Rea et al., 2013), an effect attenuated by intra-BLA administration of both the GABA_A receptor antagonist, bicuculline, and the mGluR5 receptor antagonist, MPEP, suggesting that CB₁ receptors in the BLA facilitate the expression of FCA, through a mechanism which is likely to involve the modulation of GABAergic and glutamatergic signalling. FCA was associated with increased levels of AEA in the left BLA

(side contralateral to intraplantar formalin injection). Fear-conditioned, formalin-treated rats displayed increased levels of 2-AG and PEA in the left and right BLA, respectively (Rea et al., 2013).

It is clear, therefore, that the endocannabinoid system in the amygdala, and specifically the BLA, plays an important role in mediating both unconditioned and conditioned SIA with likely interactions with GABAergic and glutamatergic signalling.

1.7.5.3 SIH:

A recent study from our group investigated the effects of repeated exposure to forced swim stress on formalin-evoked nociceptive behaviour in rats in stress normo-responsive (SD) and stress hyper-responsive (WKY) rat strains. Formalin-evoked nociceptive behaviour was increased in SD rats but decreased in WKY rats following ten days of forced swim stress (Jennings et al., 2015). AEA levels were reduced in the contralateral amygdala (relative to formalin injection) of SD rats but not WKY rats. There were also strain differences in components of the endocannabinoid system within the amygdala. For example, decreased levels of AEA and 2-AG were observed in the ipsilateral amygdala of SD, but not WKY, rats. Lower levels of CB₁ receptor mRNA were seen in the ipsilateral, but not contralateral, amygdala of WKY rats. These data indicate a role for the endocannabinoid system in the amygdala in SIH as well as implicating it in the strain differences seen in WKY and SD rats (Jennings et al., 2015). Additional studies are warranted to fully understand the role of the endocannabinoid system in the amygdala in SIH.

1.7.6 The role of the endocannabinoid system in the prefrontal cortex in pain, stress-induced analgesia and stress-induced hyperalgesia

1.7.6.1 Pain:

The PFC is involved in both the top-down descending modulation of pain and also in the affective dimension of the pain experience. Imaging studies have shown that the PFC is consistently activated by noxious stimuli (Casey et al., 1996, Davis et al., 1997, May et al., 1998, Millan, 1999, Neal et al., 1990). CB₁ receptors are expressed in the PFC (Herkenham et al., 1991, Sim-Selley et al., 2002, Tsou et al., 1998a). This, along with its projections to the PAG and amygdala (Diorio et al., 1993, Little and Carter, 2013, Marchand and Hagiwara, 1983), suggest a role for the endocannabinoid system in the PFC in pain.

As previously discussed, the mPFC is comprised of the IL, PrL and ACC. CB₁ receptors in the rodent mPFC are expressed on presynaptic GABAergic interneurons (Marsicano and Lutz, 1999, Wedzony and Chocyk, 2009). CB₁ receptors on presynaptic axon terminals face pyramidal neurons with postsynaptic mGluR5 (Lafourcade et al., 2007). A rat arthritis pain model, induced via intra-articular injections of kaolin and carrageenan through the patellar ligament, shows pain-related hyperactivity in the amygdala leading to related inhibition of mPFC pyramidal neurons (Ji and Neugebauer, 2011, Ji et al., 2010). This pain-related inhibition of mPFC neurons is due to mGluR1-mediated activation of GABA_A receptors in the mPFC which then inhibits pyramidal cell output, leading to cognitive deficits associated with persistent pain in this model (Ji and Neugebauer, 2011, Ji et al., 2010, Sun and Neugebauer, 2011). A subsequent study by these researchers investigated the effect of mGluR5 and CB₁ receptor activation on the activity of the mPFC cells in rats in the same arthritic pain model (Ji and Neugebauer, 2014). Co-activation of mGluR5 and CB₁ receptors increased mPFC activity, and inhibited pain-related neuronal activity in the amygdala in this model. Thus, there appears to be an inverse link between activation of mPFC neurons and amygdala output and a role for the endocannabinoid system in this top-down cortical control (Ji and Neugebauer, 2014, Kiritoshi et al., 2013). This group also found that group 2 mGluRs act presynaptically to inhibit glutamatergic synaptic output which may contribute to the persistent pain observed a rat model of arthritis (Kiritoshi and Neugebauer, 2015). Further evidence for a role of the endocannabinoid system in the PFC in arthritic conditions comes from work demonstrating that osteoarthritis pain is associated with increased 2-AG levels in the PFC of mice in the monosodium iodacetate model of arthritis (La Porta et al., 2015). Indeed, Kiritoshi et al., found that restoring 2-AG/ CB₁ signalling via the CB₁ agonist ACEA or MAGL inhibitor JZL184 can increase mGluR5 activity and infralimbic output and inhibit pain-related behaviour in a rat model of arthritis (Kiritoshi et al., 2016).

CB₁ receptor activity is decreased in the rostral ACC 10 days post CCI in mice, compared with sham controls (Hoot et al., 2010). CB₁ receptor levels in the rostral ACC of CCI and sham rats remained unchanged and there were no significant differences in the levels of 2-AG or AEA in the ACC between CCI and sham-operated mice. The ACC is associated with the affective component of pain (Kulkarni et al., 2005, Kuo et al., 2009, LaBuda and Fuchs, 2005, Treede et al., 1999). It is possible therefore that reduced CB₁ receptor activity in the ACC is associated with the negative affective component of neuropathic pain.

TRPV1 expression is increased in glutamatergic neurons in the mPFC (namely the PrL and IL) following spared nerve injury (SNI) (Giordano et al., 2012). Intra-PL/IL administration of AA-5-HT reduced mechanical allodynia in rats following SNI to a greater extent than that seen with a FAAH inhibitor or TRPV1 antagonist alone (Giordano et al., 2012). SNI-induced neuropathic pain is also associated with increased levels of endovanilloids and endocannabinoids in the mPFC. Intra-PrL/IL injection of AA-5-HT produced antinociceptive effects in rats more efficiently than URB597 or I-RTX, a selective FAAH inhibitor or a TRPV1 antagonist, alone (de Novellis et al., 2011b). These studies suggest that both the endocannabinoid and endovanilloid systems in the mPFC may play a role in neuropathic pain. Therapies which target these systems may prove useful in the treatment of chronic neuropathic pain.

Our group has studied the role of PPAR α in the mPFC in formalin-evoked nociceptive behaviour in rats. The PPAR α antagonist GW6471 delayed the onset of the second phase of formalin-evoked nociceptive behaviour in rats. This reduction in nociceptive behaviour was associated with a reduction in the levels of N-palmitoylethanolamide and N-oleoylethanolamide (PPAR α ligands) in the mPFC (Okine et al., 2014). Together these data suggest a facilitatory role for PPAR α in the mPFC in formalin-evoked nociceptive behaviour. Moreover, intra-ACC PEA significantly attenuated formalin-evoked nociceptive behaviour in rats, an effect attenuated by CB $_1$ receptor antagonism via AM251, but not TRPV1 antagonist via 5'iodo resiniferatoxin or PPAR α and PPAR γ antagonism via GW6471 and GW9662 respectively. Each of the antagonists, when administered alone, reduced formalin-evoked nociceptive behaviour suggesting a facilitatory role for these receptors in the ACC in inflammatory pain in rats (Okine et al., 2016). Intra-ACC PEA increased AEA levels in this region implying a possible AEA-induced activation of CB $_1$ receptors to reduce inflammatory pain-related behaviour (Okine et al., 2016).

1.7.6.2 SIA:

Lesion studies have indicated a role for the PFC in acquisition, consolidation and extinction of conditioned fear in rodents (Sierra-Mercado et al., 2006). This region has also been shown to project to other regions important in fear neurocircuitry, including the previously discussed amygdala and PAG (LeDoux, 2000, LeDoux et al., 1988, Diorio et al., 1993, Little and Carter, 2013, Marchand and Hagino, 1983). CB $_1$ receptors in the PrL cortex are involved in the amplification of panic-like aversive reactions and SIA. Thus, microinjection of bicuculline into

the rat dorsomedial and ventromedial hypothalamus induced aversive panic-like behaviour and SIA, an effect attenuated by microinjection of AM251 into the PrL (Freitas et al., 2013). This work suggests that CB₁ receptor signalling in the PrL may facilitate or augment SIA induced by stimulation of the hypothalamus. Previous work within our group has investigated the roles of CB₁ and FAAH substrates within the mPFC in FCA (Rea et al., 2018) and will be discussed in the introduction to Chapter 2 along with Chapters 3-5. Further investigation of the roles of the endocannabinoid system in the PrL, IL and ACC in SIA and FCA is warranted and is a major focus of the present thesis.

1.7.6.3 SIH:

To my knowledge there have been no published studies to date investigating the role of the endocannabinoid system in the PFC in SIH or in exacerbation of pain by negative affect and this represents the second major focus of the present thesis.

1.7.7 Clinical relevance of the prefrontal cortex:

The mPFC is a key focus of this thesis. It is a key region in the descending inhibitory pain pathway and is involved in mediating and modulating fear and FCA. fMRI has revealed loss of prefrontal grey matter and altered functional connectivity between the PFC and reciprocally connected brain regions in patients suffering from chronic pain and those seeking cognitive behavioural therapy for chronic pain (Apkarian et al., 2004, Baliki et al., 2006a, Geha et al., 2008, Moayedi et al., 2011, Kucyi et al., 2014, Jensen et al., 2012). Functional impairment of the PFC has been implicated in the pathophysiology of numerous stress-related psychiatric disorders such as PTSD, major depression, schizophrenia and generalised anxiety disorder (Liberzon and Martis, 2006, Liberzon and Phan, 2003, Ferrarelli et al., 2008, Johnstone et al., 2007, Drevets et al., 1997). The PFC is also interconnected with several other important brain regions involved in these disorders such as the hippocampus and amygdala (Laroche et al., 2000, Preston and Eichenbaum, 2013, Yu and Frank, 2015, Marek et al., 2013). Studies suggest that patients suffering from these disorders have altered functional connectivity between the PFC and these regions (Genzel et al., 2015, Vai et al., 2015, Yu et al., 2013). The subregions of the mPFC (IL, PrL and ACC) may have differential roles in pain, fear/anxiety, and pain-fear/anxiety interactions. Understanding the role of the PFC in pain and fear/anxiety interactions may allow us to better understand and treat co-morbid pain/affective disorders.

1.8 Overall hypothesis and objectives of the research presented in this thesis

The evidence reviewed in this introductory chapter suggests that the endocannabinoid system in the mPFC modulates both nociceptive and fear-related behaviour. However, there is a paucity of studies examining the role of the endocannabinoid system in the mPFC in FCA. Similarly, few studies investigating the influence of genetic background in FCA/SIA. Given the well-established role for the endocannabinoid system in pain processing, I hypothesised that (1) FAAH and MAGL substrates in the mPFC modulate pain, conditioned fear and FCA and (2) in a subregion-specific manner. Moreover, given the hyperalgesic/stress-hyper responsive phenotype of the WKY rat, I hypothesise that this strain will exhibit reduced expression of FCA compared with SD rats, and that the endocannabinoid system in the mPFC may play a role in the hyperalgesic phenotype of the WKY rat.

The main objective of the work presented herein, therefore, was to improve our understanding of the role of the endocannabinoid system in the mPFC in pain, conditioned fear and FCA. The first 4 results chapters examine the effects of FAAH (Chapter 2) or MAGL (Chapters 3-5) inhibition in the IL, PrL and ACC on formalin-evoked nociceptive behaviour, conditioned fear and FCA in rats. Chapter 6 examines the influence of genetic background (WKY rat) on FCA and associated alterations in the endocannabinoid system in the mPFC. Chapter 7 investigates the role of AEA and CB₁ in the IL of SD vs. WKY rats in the formalin test.

General aims of the present studies are:

1. To examine the effects of FAAH inhibition in the ACC in formalin-evoked nociceptive behaviour, conditioned fear and FCA.
2. To examine the effects of MAGL inhibition in the IL, PrL and ACC on formalin-evoked nociceptive behaviour, conditioned fear and FCA.
3. To investigate the influence of genetic background (by comparing SD and WKY rats) on formalin-evoked nociceptive behaviour, conditioned fear and FCA and determine associated alteration in the endocannabinoid system in the mPFC
4. To examine the role of the endocannabinoid system in the IL in formalin-evoked nociceptive behaviour in the WKY rat, a model with a depressive-like phenotype, hypersensitive to pain.

Chapter 2 : The role of fatty acid amide hydrolase substrates in the anterior cingulate cortex in formalin-evoked nociceptive behaviour, fear-conditioned analgesia and conditioned fear in the presence of nociceptive tone in rats

2.1 Introduction

The mPFC is strongly implicated in cognitive, emotional and motivational processes, and in the regulation of responses to aversion (Guimaraes et al., 2011, Laviolette et al., 2005, Baeg et al., 2001, Jiang et al., 2014, Ji et al., 2010) and pain (Okine et al., 2014, Luongo et al., 2013, Okine et al., 2016, Ji and Neugebauer, 2014, Ji and Neugebauer, 2011). In rats, the mPFC is comprised of a number of subregions that can be differentiated by anatomical connectivity and pharmacological manipulation including: the IL, PrL and ACC. In 2011, a study from our laboratory suggested a role for the PFC in pain, fear and endogenous pain suppression. Butler et al., found that rats expressing either formalin-evoked nociceptive behaviour or conditioned fear-related behaviour exhibited increased MAPK activity in the PFC, an effect not observed in rats expressing FCA (Butler et al., 2011). However, at that point in time there was a paucity of research examining the role of the specific subregions of the mPFC in FCA.

The ACC is involved in the modulation of fear behaviour (Cullen et al., 2015, Einarsson et al., 2015, Falconi-Sobrinho et al., 2017), the cognitive-affective component of pain (Johansen et al., 2001, Buchel et al., 2002), and in top-down descending modulation of pain (Calejesan et al., 2000, Fuchs et al., 2014). The ACC is connected reciprocally with both the PrL and IL and may play a role in modulating their output (Vertes, 2002).

Several imaging studies in humans, and anatomical and neurophysiological studies in rodents, have implicated a role for the ACC in pain processing (Koyama et al., 1998, Kwan et al., 2000, Sikes and Vogt, 1992, Vogt et al., 1996) and in the expression of pain-related negative emotion (Gao et al., 2004, Cao et al., 2009, Fuchs et al., 2014). Lesions in the ACC significantly reduced behavioural responding to noxious stimuli in rats (LaGraize et al., 2004, Pastoriza et al., 1996). The ACC also projects to and receives projections from the amygdala and PAG (LeDoux, 2000, Etkin et al., 2011, An et al., 1998, Little and Carter, 2013, Marchand and Hagino, 1983, Kim et al., 2011), areas shown to be involved in processing noxious and aversive stimuli, suggesting a key role for the ACC in modulating pain, fear and aversion.

Lesion studies have highlighted a role for the PFC in the acquisition, consolidation, and extinction of conditioned fear in rodents (Sierra-Mercado et al., 2006). Activation of the ACC may underlie the expression of a generalized fear memory, while inactivation of the ACC in a contextual fear-conditioning paradigm reduces fear expression in mice (Cullen et al., 2015). The role of the ACC in generalised contextual fear memory expression along with the formation of fear memory has been demonstrated by numerous studies (Einarsson et al., 2015, Li and Li, 2015, Frankland et al., 2004, Einarsson and Nader, 2012). Imaging studies have indicated a role for the ACC in response to fearful stimuli (Yoshihara et al., 2016). Blood oxygenation level dependent imaging in primary and secondary psychopaths during Pavlovian fear-conditioning revealed contrasting activity patterns in the dorsal and ventral ACC in these subjects. Primary psychopaths exhibited a pattern of activity consistent with enhanced fear expression, while secondary psychopaths exhibited a pattern of activity consistent with fear inhibition (Schultz et al., 2016). These results suggest a role for the ACC in fear expression but also in disorders where fear expression is impaired.

As mentioned previously, FCA is robust pain suppression that occurs upon re-exposure to a context previously paired with an aversive stimulus. The endocannabinoid system plays a key role in the expression of FCA (Finn et al., 2004, Butler et al., 2008, Roche et al., 2007, Ford et al., 2011, Olango et al., 2014, Olango et al., 2012, Rea et al., 2013, Corcoran et al., 2015) and unconditioned stress-induced analgesia (Connell et al., 2006, Guindon and Hohmann, 2009, Hohmann et al., 2005, Suplita et al., 2005). These studies have highlighted the importance of the endocannabinoid system in discrete brain regions including the amygdala (Rea et al., 2013), hippocampus (Ford et al., 2011) and PAG (Olango et al., 2012), all of which are connected anatomically to the mPFC. Components of the endocannabinoid system, including CB₁ receptors and the AEA-catabolising enzyme FAAH, are expressed in the mPFC (Herkenham, 1991, Herkenham et al., 1991, Mailleux and Vanderhaeghen, 1992, Mailleux et al., 1992, Tsou et al., 1998a, Moldrich and Wenger, 2000, Oropeza et al., 2007, Egertova et al., 2003, Rubino et al., 2008b, de Novellis et al., 2011b).

Recent work from our group has demonstrated a role for the endocannabinoid system in the mPFC in FCA. The FAAH inhibitor URB597 has opposing effects following microinjection into the rat IL or PrL, enhancing FCA in the PrL but attenuating it in the IL. In contrast, the CB₁ receptor antagonist/inverse agonist AM251 attenuates FCA following microinjection into both regions (Rea et al., 2018). The role of the endocannabinoid system in the ACC in fear, formalin-evoked nociceptive behaviour and FCA has not yet been examined.

This chapter will investigate the role of the endocannabinoid system in the ACC in formalin-evoked nociceptive behaviour, conditioned fear and FCA with the following hypothesis: FAAH inhibition in the ACC will attenuate the expression of FCA and increase the expression of formalin-evoked nociceptive behaviour while having no effect on the expression of contextual fear in the presence of nociceptive tone. Therefore, the aims of the study described in this chapter were:

- To determine the role of the endocannabinoid system in the ACC in formalin-evoked nociceptive behaviour, expression of fear behaviour in the presence of formalin-evoked nociceptive tone and expression of FCA. This was achieved by examining the effects of intra-ACC administration of the FAAH inhibitor, URB597, or the CB₁ receptor antagonist/inverse agonist, AM251, on formalin-induced nociceptive behaviour, fear-related behaviour in the presence of nociceptive tone and FCA in rats.
- To examine if behavioural changes are associated with alterations in endocannabinoid levels in the ACC using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

2.2 Materials and Methods

2.2.1 Animals:

Male Lister-Hooded rats (260g – 320g at time of testing Charles River, Margate, Kent, UK) were used in this study. Before surgery, animals were housed 2-3 per flat bottomed cage (L: 45 x H: 20 x W: 20cm) containing wood shavings as bedding (LBS, Surrey, UK). Animals were kept at a constant temperature ($21^{\circ}\text{C} \pm 2^{\circ}\text{C}$) under standard lighting conditions (12:12h light–dark, lights on from 0800 to 2000h). Experiments were carried out during the light phase between 0800 and 1700h. Food (14% Harlan-Teklad-2014 Maintenance Diet, Harlan Laboratories, Belton, Loughborough, UK) and water were provided *ad libitum*. The experimental protocol was carried out after approval by the Animal Care and Research Ethics Committee, National University of Ireland Galway, under license from the Health Products Regulatory Authority and in compliance with the European Communities Council directives 86/609 and 2010/63 and conformed to the ARRIVE guidelines.

2.2.2 Cannulae implantation:

Animals were left to acclimatize for 4-8 days after delivery before surgery. Animals were placed under isoflurane (2-3% in O₂, 0.5L/min) and stainless steel guide cannulae (5mm length, 22G, Bilaney Consultants, Sevenoaks, UK) were stereotaxically implanted 1mm above the left and right ACC (coordinates: AP = +1.0mm from bregma, RML = -1.3mm, LML = -1.5mm at an angle of 12°, DV: -1.3mm from the meningeal dura matter according to the rat brain atlas (Paxinos and Watson, 1998)). The cannulae were permanently fixed to the skull using stainless steel screws (0.8 x 1.25mm, Bilaney Consultants, Sevenoaks, UK) and carboxylate cement (Durelon Powder Fast Setting and Durelon Liquid, Dental Medical Ireland, Dublin, Ireland). A stylet made from stainless steel tubing (5mm length, Plastics One Inc., Roanoke, VA, USA) was inserted into the guide cannula to prevent blockage by debris. The nonsteroidal anti-inflammatory agent carprofen (5mg/kg, s.c., Rimadyl; Pfizer, Kent, UK), and the broad-spectrum antibiotic enrofloxacin (2.5mg/kg, s.c., Baytril; Bayer Ltd., Dublin, Ireland), were administered to manage postoperative pain and to prevent infection, respectively. Following surgery, animals were singly housed and a single daily dose of enrofloxacin (2.5mg/kg, s.c., Baytril; Bayer Ltd., Dublin, Ireland) was administered for the following three days. A minimum of 6 days was allotted to allow rats to recover before any experimentation

commenced. During this period, the rats were handled daily, stylets checked, and their body weight and general health monitored.

2.2.3 Chemicals and drug preparation:

The CB₁ receptor antagonist/inverse agonist AM251 [(N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3 carboxamide) Sigma-Aldrich, Dublin, Ireland] and the fatty acid amide hydrolase (FAAH) inhibitor and indirect agonist URB597 [(30-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate) Sigma-Aldrich, Dublin, Ireland] were prepared from a 4mM stock to a working concentration of 2mM/0.3µl DMSO (dimethyl sulfoxide, 100%; Sigma-Aldrich, Dublin, Ireland) and from a 0.2mM stock to a working concentration of 0.1mM/0.3µl DMSO, respectively. A solution of both AM251 and URB597 combined (2mM AM251/0.1mM URB597 in 0.3µl DMSO) was also prepared from these stock concentrations. These concentrations of URB597 and AM251 are based on previous work carried out by our laboratory and evidence from the literature which indicated *in vivo* efficiency in rat models of pain, fear or FCA (Lisboa et al., 2010, Ford et al., 2011, Freitas et al., 2013, Rea et al., 2018, Hohmann et al., 2005).

2.2.4 Experimental procedure:

The FCA procedure was essentially as described previously (Butler et al., 2008, Finn et al., 2004, Ford et al., 2011, Olango et al., 2012, Rea et al., 2009, Roche et al., 2010, Roche et al., 2007). The experiment consisted of 2 phases, conditioning and testing, occurring 24 hours apart. Rats were randomly assigned to groups and the sequence of testing was randomized while ensuring testing occurred 6-7 days following surgery.

The conditioning phase involved placing the rats in a Perspex fear-conditioning / observation box (L:30 x W:30 x H:40cm) at 30 lux, and after 15 seconds they received the first of 10 footshocks (0.4mA, 1s duration; LE85XCT Programmer and Scrambled Shock Generator, Linton Instrumentation, Norfolk, UK) spaced 60 seconds apart. 15 seconds following the final footshock, the rats were returned to their home cage. Control rats not receiving footshock were exposed to the arena for an equivalent 10-minutes. The arena was cleaned using 0.5% acetic acid after each rat.

The test phase occurred 23.5 hours after the conditioning phase. Rats received an intraplantar injection of 50µl formalin (2.5% formalin solution prepared in sterile saline) into the right hind paw while under brief isoflurane anaesthesia (~1.5min; 2% in O₂; 0.5L/min). Formalin-induced oedema was assessed by measuring the change in diameter of the right hind paw immediately before and 60 minutes after formalin administration using Vernier callipers. Rats were returned to their home cage for 15 minutes following formalin injection after which they received intra-ACC microinjection of DMSO vehicle (0.3µl DMSO), URB597 (0.1mM/0.3µl DMSO), AM251 (2mM/0.3µl DMSO) or a combination of both AM251 and URB597 (2mM AM251/0.1mM URB597/0.3µl DMSO) into the left and right ACC. An injection needle was inserted through the guide cannula into the ACC and protruded 1mm beyond the tip of the pre-implanted guide cannula. A volume of 0.3µl was injected for 60s using a 1µl Hamilton microsyringe attached to polyethylene tubing and a Harvard PHD2000 infusion pump (Harvard Apparatus, Kent, UK). The injector was left for another 60s before removal to allow adequate drug infusion. Rats were returned to their home cage for 15 minutes before re-exposure to the arena used in the conditioning phase for 30-minutes as done in previous studies (Rea et al., 2013, Butler et al., 2012, Olango et al., 2014). The arena was cleaned using 0.5% acetic acid after each rat. See Figure 2.1 for graphical representation of the experimental procedure.

This design resulted in 8 experimental groups (Starting n=12 per group for surgery; final n=7-12 per group for data analysis) as illustrated in Table 2.1. A bat detector (Batbox Duet, Batbox, Steyning, West Sussex, UK), positioned beside the Perspex arena, was used to detect vocalisation in the 22kHz range and output recorded using a digital video recorder. Animal behaviour was recorded over this 30-minute timeframe using a video camera located beneath the observation chamber with video feed from this camera recorded by digital video recorder for subsequent behavioural analysis.

After 30-minutes in the arena, rats were removed and euthanised by decapitation. Brains were rapidly removed within a 2-minute timeframe, snap-frozen on dry ice and stored at -80°C. After decapitation, 0.3µl of 2% fast green dye (dissolved in DMSO) was also injected into the left and right ACC for post-mortem confirmation of microinjection sites during cryo-sectioning.

Group	Conditioning	Formalin i.pl.	Drug/Vehicle	<i>n</i>
1	FC	Formalin	Vehicle	12
2	NFC	Formalin	Vehicle	8
3	FC	Formalin	AM251	9
4	NFC	Formalin	AM251	11
5	FC	Formalin	URB597	8
6	NFC	Formalin	URB597	9
7	FC	Formalin	URB597+AM251	8
8	NFC	Formalin	URB597+AM251	7

Table 2.1: Summary of experimental groups: i.pl., intra-plantar; FC, fear-conditioned; NFC, non – fear-conditioned

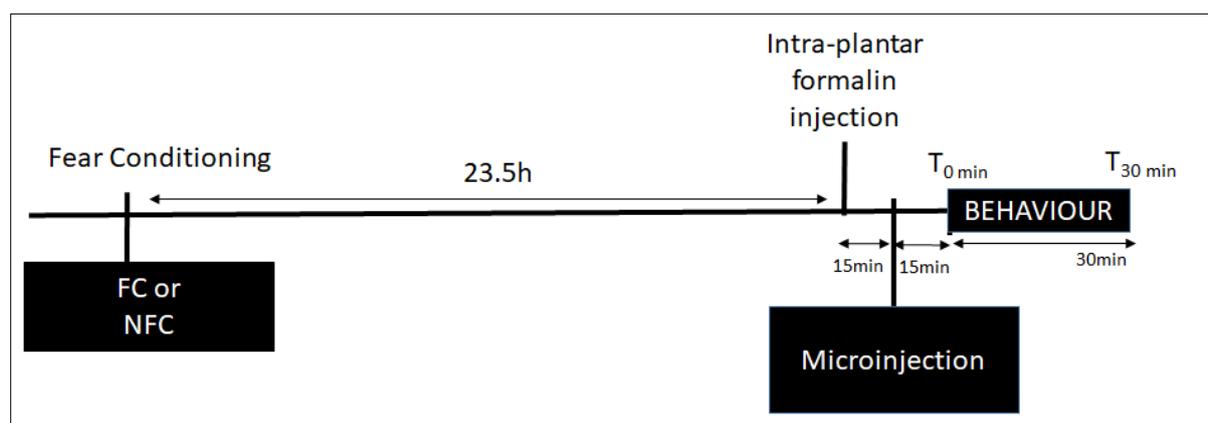


Figure 2.1: Graphical representation of the experimental procedure.

2.2.5 Brain removal:

Following decapitation, an incision was made using a scissors along the top of the head and the skin pulled back to expose the skull. The optic ridge between the eyes and the back of the skull was broken with rongeurs. Using a scissors, a cut was made carefully along the midline of the skull from the back, maintaining pressure away from the brain surface, and the parietal and frontal skull was removed. The remaining bone along the sinus between the olfactory bulbs and frontal cortex was carefully removed, as was the bone over the nasal cavity and eye socket. The dura mater was removed, the trigeminal nerve was cut, and the brain removed from the skull using curved forceps. The brains were snap frozen on dry ice and stored at - 80°C.

2.2.6 Behavioural analysis:

Ethovision XT 8.0 software package (Noldus, Wageningen, The Netherlands) was used to analyse behaviour, allowing for continuous event recording over each 30-minute trial. The behaviours assessed were, fear behaviour including the duration of freezing (defined as the cessation of all visible movement except that necessary for breathing) and the duration of 22kHz ultrasonic vocalization; general motor behaviours including walking, rearing, and grooming and nociceptive behaviours (composite pain score (CPS)). Nociceptive behaviours were measured using the weighted composite pain scoring technique (Watson et al., 1997). Nociceptive behaviours are divided into two types according to this method; the first is time spent elevating the formalin-injected paw without contact with any other surface (pain 1). The second is the spent holding, licking, biting, shaking or flinching the formalin injected paw (pain 2). The composite pain score equation is given as Composite Pain Score [CPS = (duration of pain 1 + duration of pain 2) / (total duration of analysis period)] (Watson et al., 1997). The Ethovision system automatically tracked total distance moved.

2.2.7 Histological verification of intracerebral injection sites:

Stereotaxic coordinates were verified histologically on 2 animals before the start of the study. Frozen coronal brain sections were cut at 150µm thickness on a cryostat at -20°C from the start of the mPFC (Bregma 3.7mm) through to the end (Bregma -0.26mm) with reference to the rat brain atlas (Paxinos and Watson, 1998). Sections containing fast green dye were mounted on gelatinised superfrost glass slides and counterstained with cresyl violet. This allowed for greater accuracy in determining the microinjection location when viewed both by eye and under a light microscope.

2.2.8 Tissue isolation by Palkovits punch:

Frozen coronal brain sections were collected at 150µm on a cryostat at -20°C. Slices were collected on superfrost glass slides and microinjection site positions were determined and noted on photocopied images from a rat brain atlas (Paxinos and Watson, 1998) prior to data analysis. Tissue was punched from the frozen 150µm coronal sections using cylindrical brain punchers (Harvard Apparatus, Massachusetts, USA; internal diameters 0.5 and 2mm). Tissue from the left and right ACC (Bregma 3.70 to 2.20mm, 0.5mm puncher and Bregma 1.70 to -0.26mm, 2mm puncher) was punched. Punched tissue was kept frozen throughout the collection

procedure. After collection, punches were placed in labelled 1.5ml microfuge tubes (Sarstedt, Nümbrecht, Germany), tissue weight recorded, and stored at -80°C.

2.2.9 Measurement of levels of endocannabinoids in the anterior cingulate cortex:

Quantification of endocannabinoids and fatty acid amides in ACC tissue punches was carried out using a lipid extraction method described previously (Ford et al., 2011, Kerr et al., 2012, Olango et al., 2012, Rea et al., 2013, Rea et al., 2014b). This method was modified slightly to consider low tissue weights and endocannabinoid levels. Punched tissue was homogenized for ~3 seconds using an ultrasonic homogeniser/sonicator and placed immediately on ice (Mason, Dublin, Ireland) in 200µl of 100% acetonitrile containing fixed amounts of deuterated internal standards (50ng d8-2-AG, and 2.5ng d8-AEA, d2-OEA and d4-PEA). Following homogenisation, samples were centrifuged at 4°C for 15 minutes at 14,000g (Hettich® centrifuge Mikro 22R, Hettich, Germany). Once the centrifugation step was complete, 180µl of supernatant was collected from each sample and placed in newly labelled 1.5ml microfuge tubes. These new samples, together with tubes containing the standard curve (see below), were then evaporated until dry in a centrifugal evaporator (Savant™ SPD131DDA SpeedVac™ Concentrator Thermo Scientific, City, USA). Lyophilised samples and standards were resuspended in 40µl 65% acetonitrile.

A 10-point standard curve was prepared. 10 1.5ml microfuge tubes were labelled 10 to 1 and 75µl of 100% acetonitrile were added to each. The highest standard (standard 10) was then created by adding 25µl of 100% acetonitrile containing known fixed amounts of undeuterated internal standards (125ng 2-AG and 12.5ng AEA, OEA and PEA). Samples were vortexed for 20 seconds and 25µl of this mixture was removed and placed in next Eppendorf in sequence (moving from highest: standard 10 to lowest: standard 1). This process of serial dilution was repeated for each of the standards until the lowest standard, standard 1 was reached. 25µl of mixture was also removed from standard 1 and discarded to ensure all standards contain the same volume of liquid. 200µl of 100% acetonitrile containing known fixed amounts of deuterated internal standards (50ng d8-2-AG, and 2.5ng d8-AEA, d2-OEA and d4-PE) was added to each standard before vortexing each microfuge tube again.

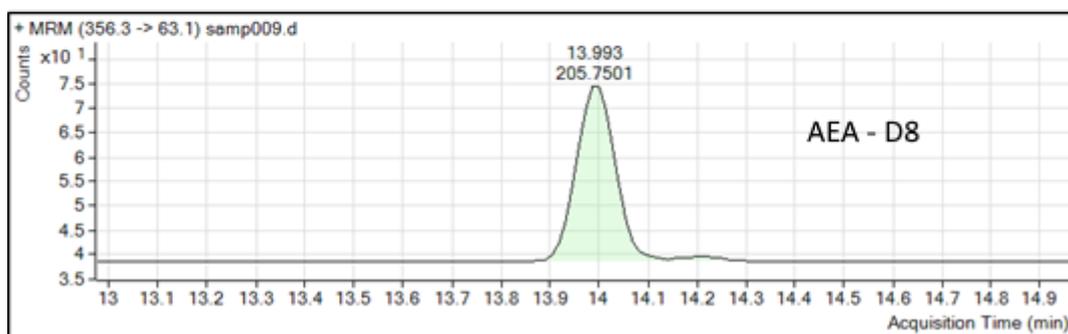
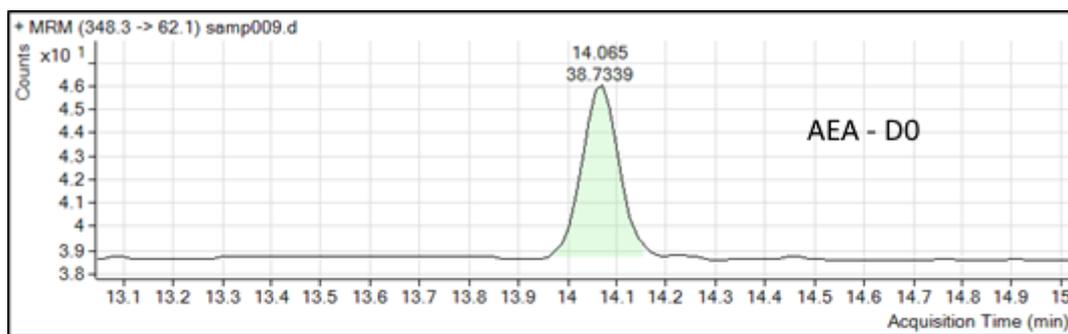
Liquid chromatography-tandem mass spectrometry was then performed to allow for simultaneous measurement of endocannabinoids and fatty acid amides. 8µl of each sample was injected onto a Zorbex SB C18 column (Agilent Santa Clara, California, USA) having length,

internal diameter and particle size dimensions of 50mm, 2.1mm and 1.8µm respectively for chromatographic separation. Solution A (high-performance liquid chromatography-grade water with 0.1% formic acid) and solution B (100% acetonitrile with 0.1% formic acid) comprised the mobile phases which had a flow rate of 300µl/min. A reversed phase gradient elution was used, comprising of 45% solution B for the first minute, then linearly increased to 100% until 5 minutes into the run and maintained at 100% solution B until the assay run finished at 12 minutes. A further 4.1 minutes was required to re-equilibrate the column at 45% solution B before the next injection. Analyte detection was carried out in electrospray positive ionisation mode on an Agilent 1200 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies, Cork, Ireland).

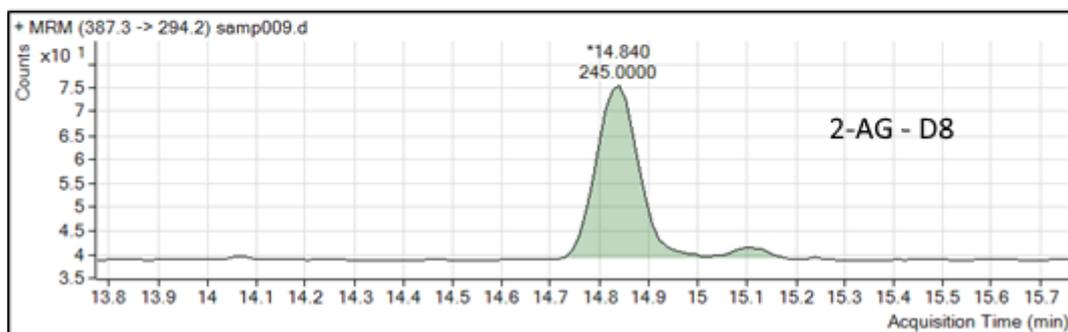
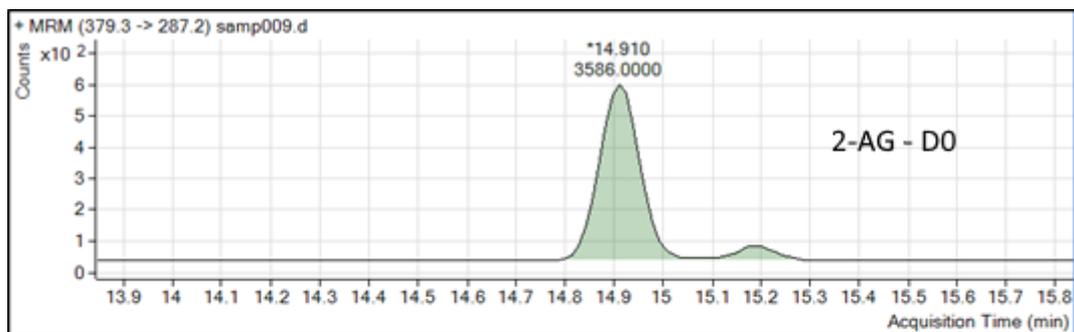
Ratiometric quantification was carried out using the Agilent MASSHUNTER Quantitative Analysis Software (Agilent Technologies, Cork, Ireland). This software allows for the creation of a standard curve from the peak area of each undeuterated standard against its corresponding deuterated internal standard. The amount of analyte in each unknown sample was then calculated from this standard curve of Relative response vs. Relative concentration i.e. $(\text{Peak Area}_{\text{analyte(undeuterated)}} / \text{Peak area}_{\text{analyte(deuterated)}}) \text{ vs } (\text{Concentration}_{\text{analyte(undeuterated)}} / \text{Concentration}_{\text{analyte(deuterated)}})$. The limits of quantification were 1.32pmol/g AEA, 12.1pmol/g 2-AG, 1.5pmol/g PEA and 1.41pmol/g OEA.

Sample chromatograms for AEA, 2-AG, PEA and OEA along with a sample 10-point calibration curve are shown below (Figure 2.2 and Figure 2.3)

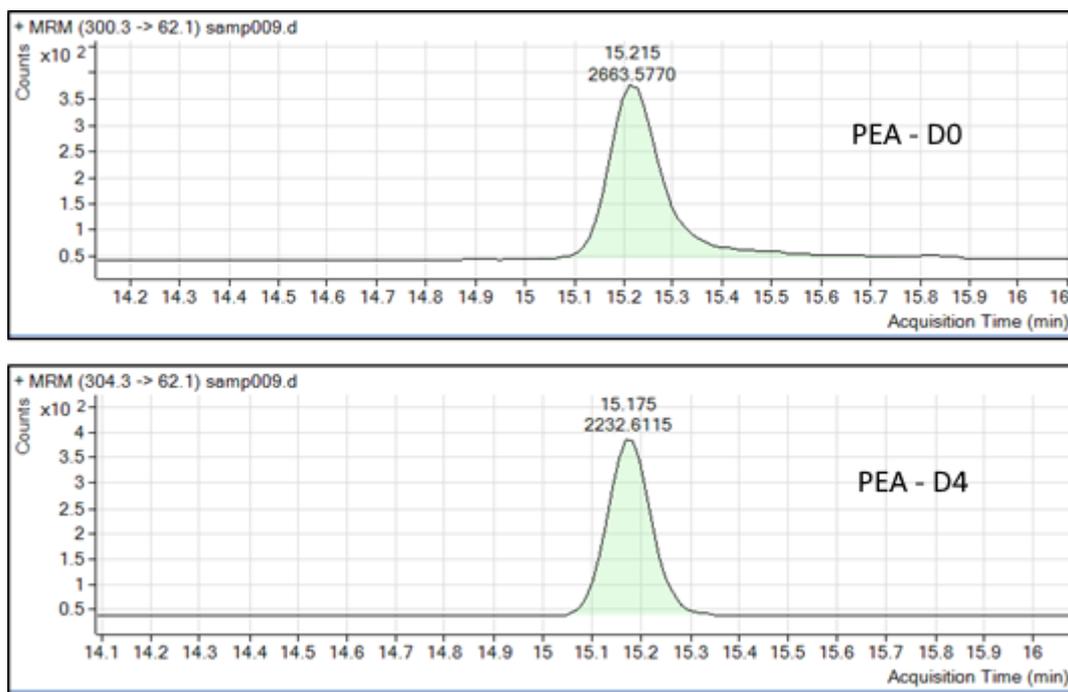
A) AEA



B) 2-AG



C) PEA



D) OEA

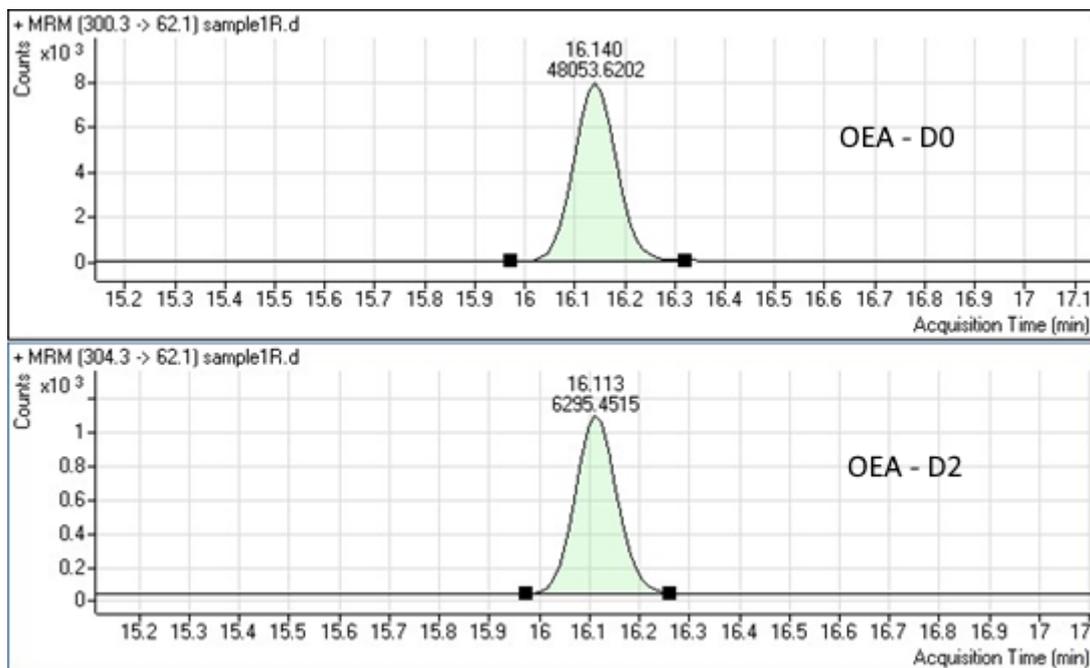


Figure 2.2: Chromatograms of endocannabinoids and related N-acylethanolamines. Top image displays the undeuterated target analytes and bottom image is the deuterated internal standard. A) AEA: Anandamide; B) 2-AG: 2-Arachidonoylglycerol; C) PEA: *N*-palmitoylethanolamide; D) OEA: *N*-oleoylethanolamine.

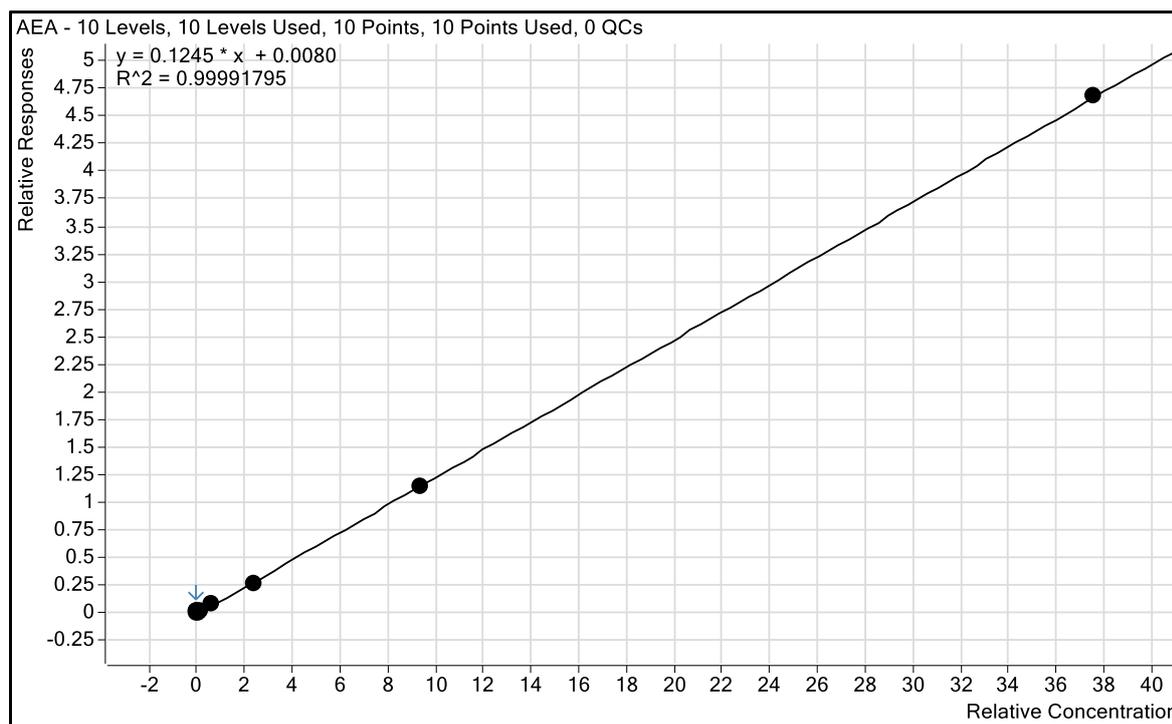


Figure 2.3: Sample 10-point calibration curve of AEA constructed from the standard curve. Plot of Relative Response vs. Relative Concentration for AEA. Relative response on the y-axis is the ratio of peak area of undeuterated analyte to peak area of deuterated analyte; whereas, relative concentration on the x-axis is the ratio of amount in ng of undeuterated analyte to the amount in ng of deuterated analyte.

2.2.10 Statistical analysis:

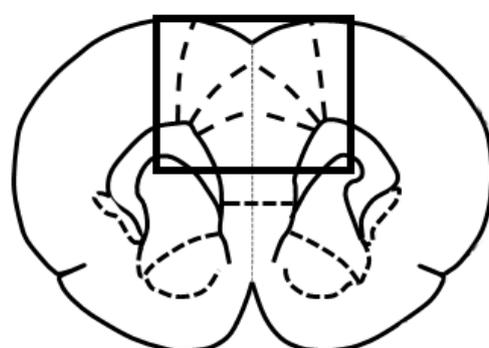
The IBM SPSS statistical software package (SPSS v23.0 for Microsoft Windows; Chicago, IL, USA) was used to analyse all data. Normality and homogeneity of variance were assessed using Shapiro-Wilk's and Levene's test, respectively. All data except the duration of freezing, 22kHz ultrasonic vocalisation and defecation were found to be parametric. Behavioural data and mass spectrometry data were analysed by 2-way analysis of variance (ANOVA), with fear-conditioning and drug treatment as factors. Time course behavioural data were analysed by 2-way repeated measures ANOVA with time as the within-subjects factor and fear-conditioning and drug treatment as the between-subjects factors. Sphericity was tested using Mauchly's Test for Sphericity. If sphericity was violated a Greenhouse-Geisser correction was used. Post-hoc pairwise comparisons were made with Tukey's test when appropriate. For data that were not normally distributed, non-parametric statistics were performed. Kruskal Wallis analysis of variance by rank was performed followed by Dunn-Bonferroni pairwise comparisons if

appropriate. For repeated measures non-parametric data Kruskal Wallis was performed followed by Dunn-Bonferroni post-hoc where appropriate. Data were considered significant when $p < 0.05$. Results are expressed as group means \pm standard error of the mean (SEM) or median with interquartile range.

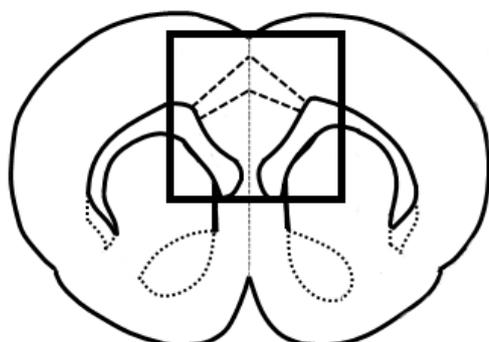
2.3 Results

2.3.1 Histological verification of microinjection sites

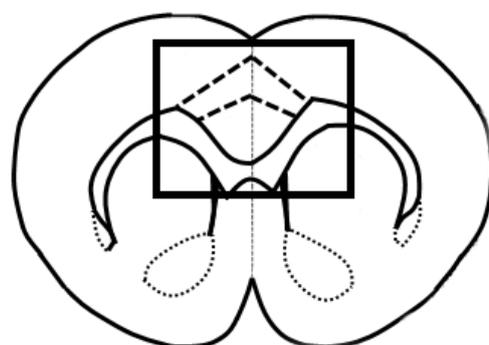
After histological verification, 75% of the microinjections carried out as part of this experiment were found to be within the borders of the left and right ACC (Figure 2.4). The remaining 25% were placed just above the ACC in the motor cortex (M2 area) or in the corpus callosum. The data analysed were derived only from rats where intra-cerebral injections were accurately placed in the ACC.



+ 2.2 mm from Bregma



+ 1.7 mm from Bregma



+ 1.6 mm from Bregma

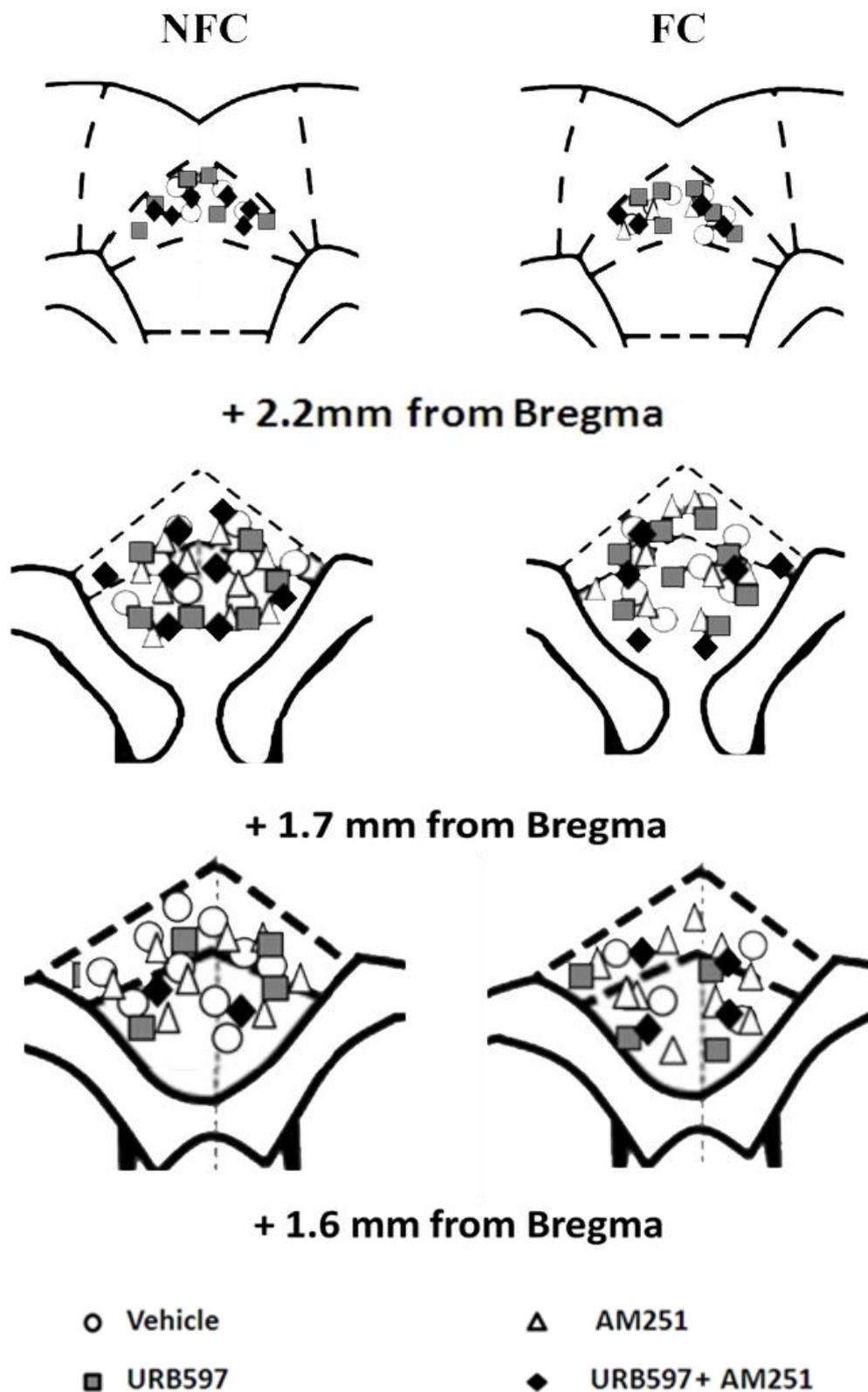


Figure 2.4: Histological verification of injector site location. Adapted from Paxinos & Watson (1998). NFC: Non-fear-conditioned; FC: Fear-conditioned; ACC: anterior cingulate cortex.

2.3.2 Effects of intra-ACC administration of AM251, URB597 or URB597+AM251 on formalin-evoked nociceptive behaviour and FCA

Intra-plantar injection of formalin induced a robust nociceptive response in the form of licking, biting, shaking, flinching and elevation of the injected paw. Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,64)=0.003$, $p=0.953$), treatment ($F(3,64)=0.327$, $p=0.806$) or fear-conditioning*treatment ($F(3,64)=0.591$, $p=0.623$) on the change in hind paw diameter pre- versus post-formalin injection (Figure. 2.5).

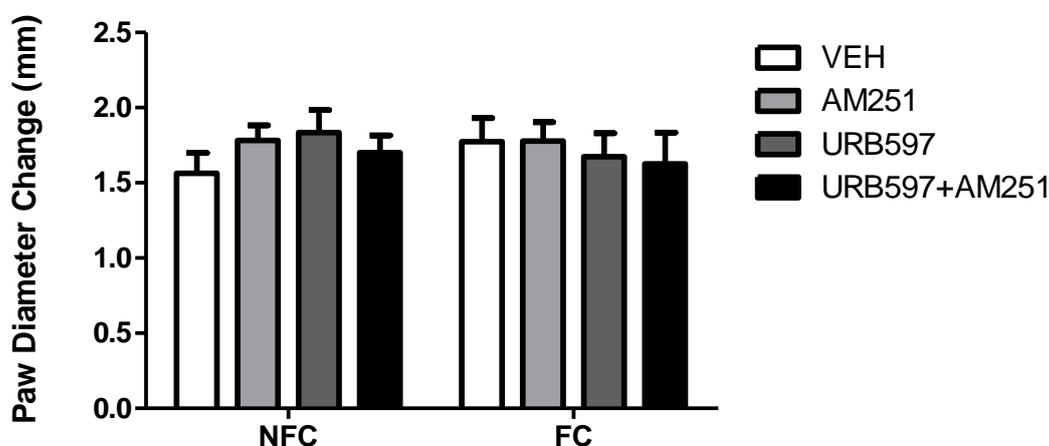


Figure 2.5: Effects of fear-conditioning and bilateral administration of URB597, AM251 or URB597+AM251 directly into the ACC on paw diameter change (mm) pre and post formalin injection (oedema). All data are expressed as mean \pm SEM ($n=7-12$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Two-way ANOVA revealed a significant main effect of fear-conditioning ($F(1,64)=84.125$, $p<0.001$), but not treatment ($F(3,64)=1.190$, $p=0.321$) or fear-conditioning*treatment ($F(1,64)=1.429$, $p=0.243$) on CPS over the entire 30-minute testing period. Re-exposure of rats to the arena previously paired with footshock resulted in a significant reduction in formalin-evoked nociceptive behaviour, confirming the expression of FCA (FC VEH vs. NFC VEH, $p<0.01$). Neither AM251, URB597 nor a combination of URB597+AM251 had any significant effect on formalin-evoked nociceptive behaviour or FCA (Figure 2.6).

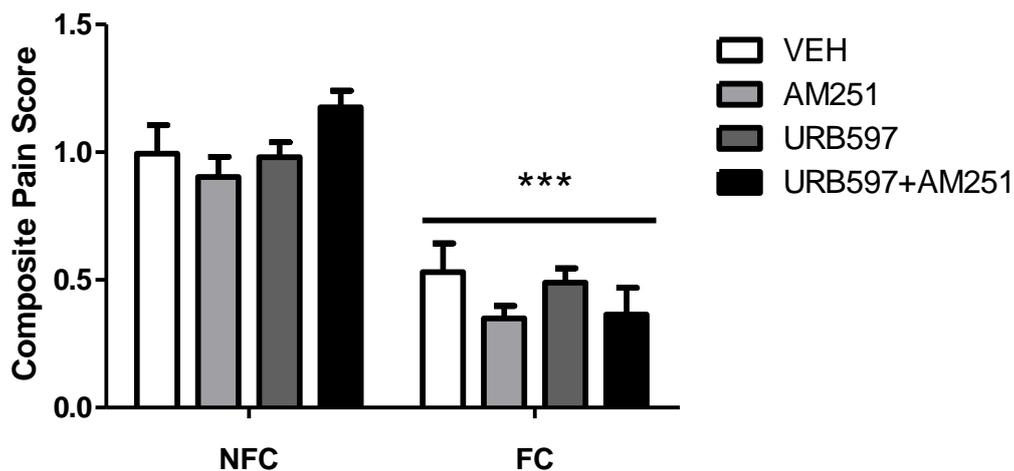


Figure 2.6: Effects of fear-conditioning and bilateral administration of URB597, AM251 or URB597+AM251 directly into the ACC on formalin-evoked nociceptive behaviour in rats over the full 30-minute testing period. *** $p < 0.001$ vs. NFC (Tukey's). All data are expressed as mean \pm SEM ($n = 7-12$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Temporal analysis of the data subdivided into 10-minute time bins and using 2-way repeated measures ANOVA revealed a significant main effect of fear-conditioning ($F(1,64) = 84.125$, $p < 0.001$) but not treatment ($F(3,64) = 1.190$, $p = 0.321$) or fear-conditioning*treatment ($F(1,64) = 1.429$, $p = 0.243$). Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated ($\chi^2(2) = 6.826$, $p < 0.05$) and therefore a Greenhouse-Geisser correction was used. There was a significant effect of time ($F(1.81, 116.08) = 99.34$, $p < 0.001$), time*fear-conditioning ($F(1.81, 116.08) = 10.95$, $p < 0.001$), but not time*treatment ($F(5.44, 116.08) = 1.74$, $p = 0.124$) or time*fear-conditioning*treatment ($F(5.44, 116.08) = 1.17$, $p = 0.326$) on CPS. FCA was expressed for the first 20 minutes following re-exposure to the conditioned arena (FC VEH vs. NFC VEH; 0-10mins, $p < 0.05$; 10-20mins, $p < 0.01$). Neither AM251, URB597 nor a combination of URB597+AM251 had any significant effect on formalin-evoked nociceptive behaviour or FCA (Figure 2.7).

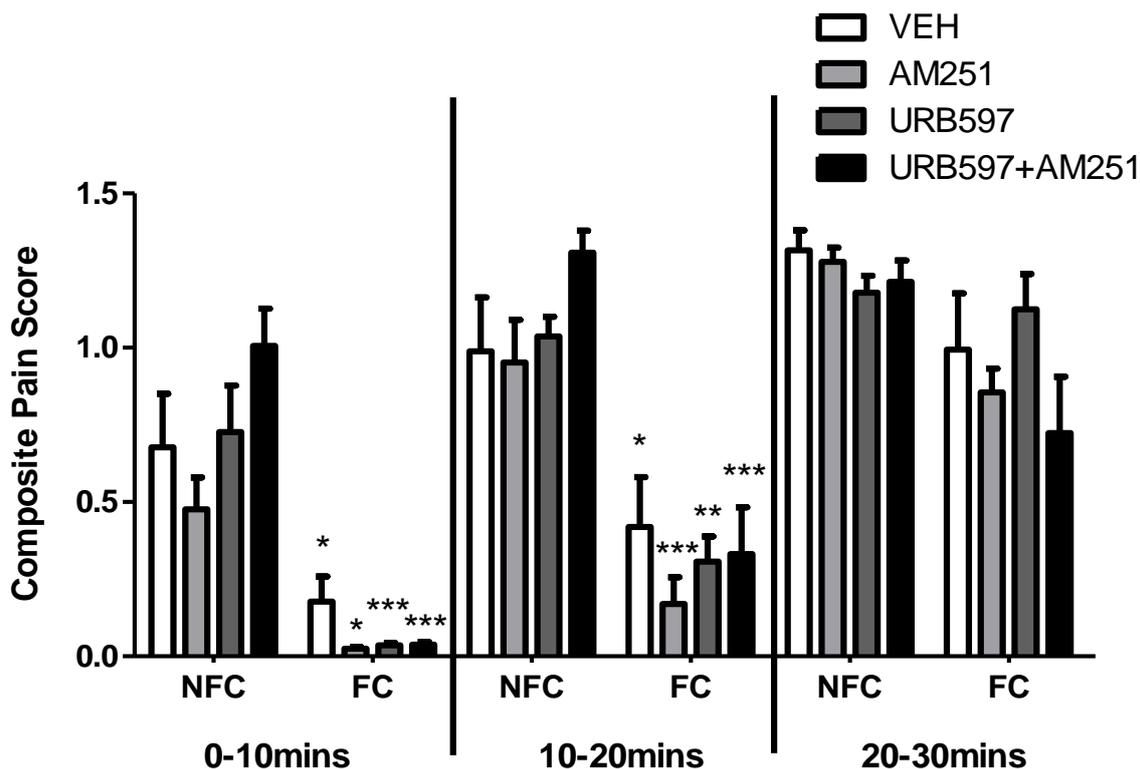


Figure 2.7: Effects of fear-conditioning and bilateral administration of URB597, AM251 or URB597+AM251 directly into the ACC on formalin-evoked nociceptive behaviour in rats over the full 30-minute testing period subdivided into 10-minute time bins. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ FC vs. NFC (Tukey's). All data are expressed as mean \pm SEM ($n = 7-12$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

2.3.3 Effects of intra-ACC administration of AM251, URB597 or URB597+AM251 on expression of conditioned fear behaviour in the presence of formalin-evoked nociceptive tone

Duration of Freezing:

Kruskal Wallis comparisons revealed a significant main effect on the duration of freezing (s) ($\chi^2(7) = 55.135$, $p < 0.001$) over the course of the 30-minute testing period. Further post-hoc analysis using Dunn-Bonferroni revealed that re-exposure of rats to the arena previously paired with footshock resulted in a significant increase in the duration of freezing (FC VEH vs. NFC VEH, $p < 0.05$) over the course of the 30-minute testing period. Neither AM251, URB597 nor

a combination of URB597+AM251 had any significant effect on the duration of freezing expressed (Figure 2.8).

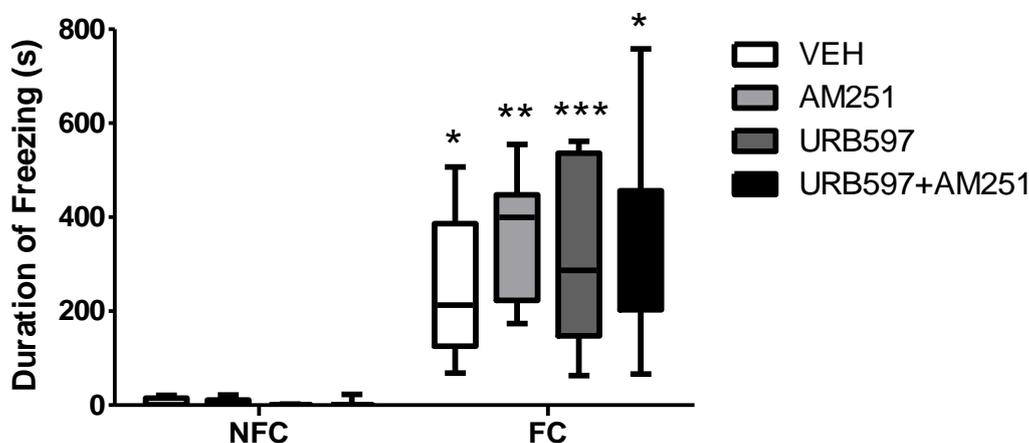


Figure 2.8: Effects of fear-conditioning and bilateral administration of URB597, AM251 or URB597+AM251 directly into the ACC on the duration of freezing in formalin-injected rats over the full 30-minute testing period. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs. NFC; (Dunn-Bonferroni). All data are expressed as median with interquartile range ($n = 7-12$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Analysis of the data subdivided into 10-minute time bins using Kruskal-Wallis revealed a significant between-group effect on the expression of freezing at 0-10mins ($\chi^2(7) = 56.064$, $p < 0.001$), 10-20mins ($\chi^2(7) = 27.417$, $p < 0.001$) but not 20-30mins ($\chi^2(7) = 4.603$, $p = 0.708$). Post-hoc analysis using the Dunn-Bonferroni test revealed that re-exposure of rats to the arena previously paired with footshock resulted in a significant increase in the duration of freezing during the first 10-minutes of the trial (FC VEH vs. NFC VEH; 0-10mins, $p < 0.05$). Neither AM251, URB597 nor a combination of URB597+AM251 had any effect on the duration of freezing (Figure 2.9).

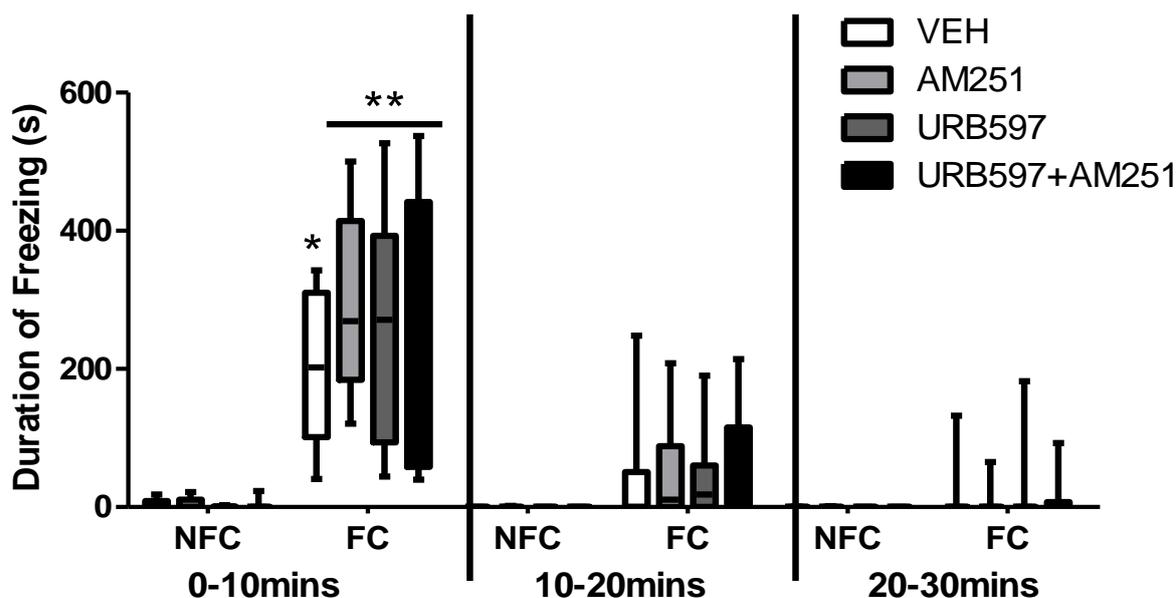


Figure 2.9: Effects of fear-conditioning and bilateral administration of URB597, AM251 or URB597+AM251 directly into the ACC on the duration of freezing in formalin-injected rats over the full 30-minute testing period, subdivided into 10-minute time bins. * $p < 0.05$, ** $p < 0.01$ FC vs. NFC; (Dunn-Bonferroni). All data are expressed as median with interquartile range ($n = 7-12$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Duration of 22kHz Ultrasonic Vocalisation:

Kruskal Wallis comparisons revealed a significant main effect on the duration of 22kHz ultrasonic vocalization ($\chi^2(7) = 53.06$, $p < 0.001$) over the course of the 30-minute testing period. Further post-hoc analysis using Dunn-Bonferroni revealed that re-exposure of rats to the arena previously paired with footshock resulted in a significant increase in the duration of 22kHz ultrasonic vocalization (FC VEH vs. NFC VEH, $p < 0.05$) over the course of the 30-minute testing period. Neither AM251, URB597 nor a combination of URB597+AM251 had any significant effect on the duration of ultrasonic vocalisation (Figure 2.10)

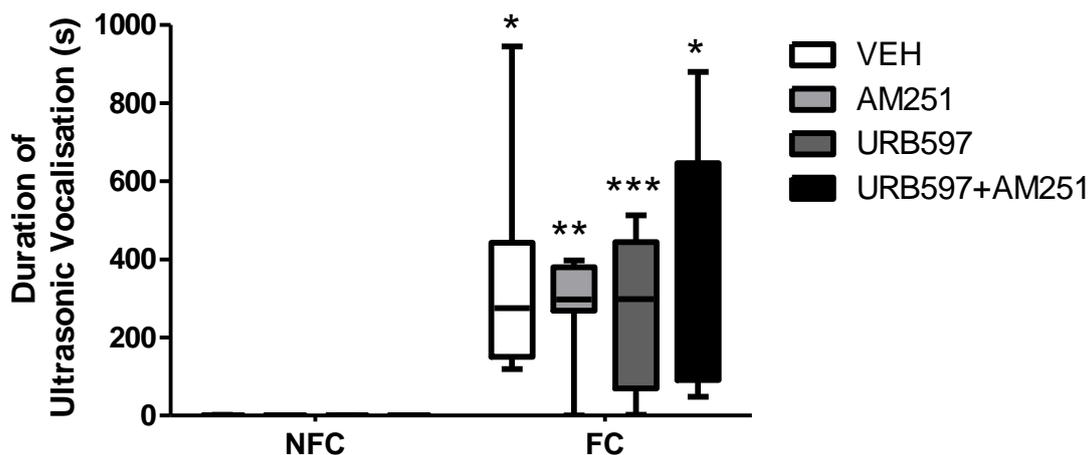


Figure 2.10: Effects of fear-conditioning and bilateral administration of URB597, AM251 or URB597+AM251 directly into the ACC on the duration of 22kHz ultrasonic vocalisation in formalin-injected rats over the full 30-minute testing period. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. NFC; (Dunn-Bonferroni). All data are expressed as median with interquartile range ($n = 7-12$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Analysis of the data subdivided into 10-minute time bins using Kruskal-Wallis revealed a significant between-group effect on the expression of 22kHz ultrasonic vocalisation at 0-10mins ($\chi^2(7) = 50.474$, $p < 0.001$), 10-20mins ($\chi^2(7) = 15.140$, $p < 0.05$) but not 20-30mins ($\chi^2(7) = 6.875$, $p = 0.442$). Post-hoc analysis using the Dunn-Bonferroni test revealed that re-exposure of rats to the arena previously paired with footshock resulted in a significant increase in the duration of 22kHz ultrasonic vocalisation for the first 10-minutes of the testing period (FC VEH vs. NFC VEH, 0-10mins, $p < 0.05$). Neither AM251, URB597 nor a combination of URB597+AM251 had any significant effect on the duration of ultrasonic vocalisation (Figure 2.11).

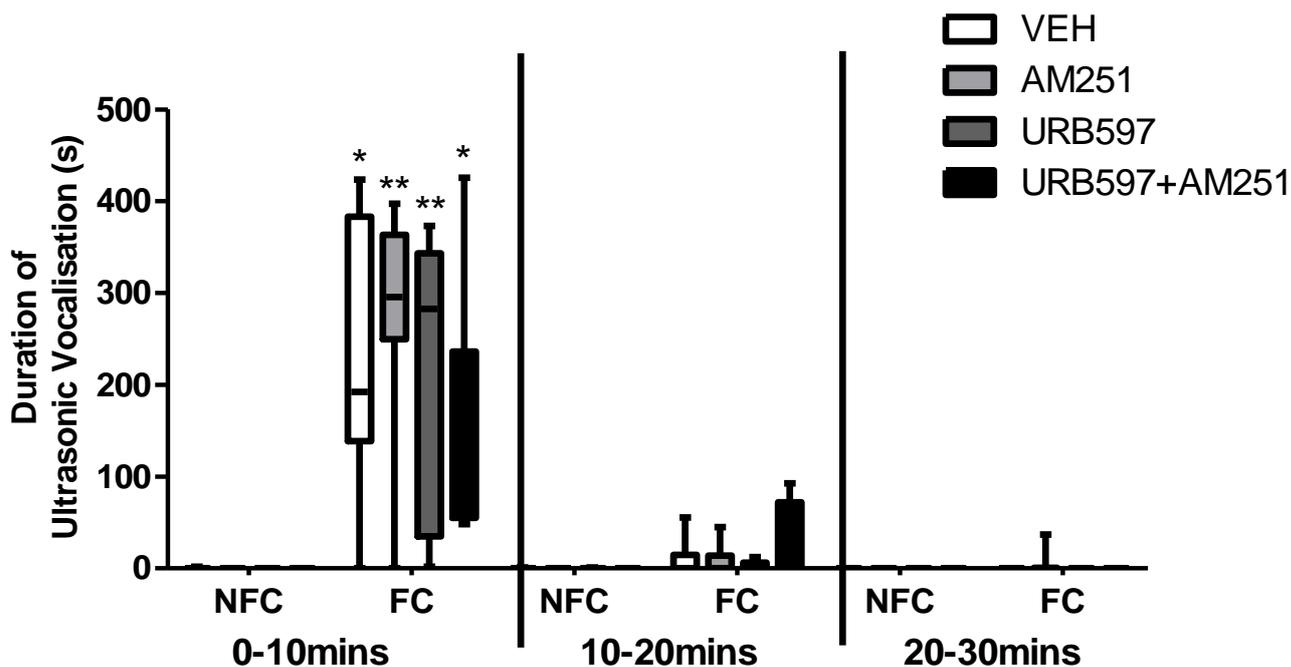


Figure 2.11: Effects of fear-conditioning and bilateral administration of URB597, AM251 or URB597+AM251 directly into the ACC on the duration of 22kHz ultrasonic vocalisation in formalin-injected rats over the full 30-minute testing period, subdivided into 10-minute time bins. * $p < 0.05$, ** $p < 0.01$, FC vs. NFC (Dunn-Bonferroni). All data are expressed as median with interquartile range ($n = 7-12$ per group). NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

2.3.4 Effects of fear-conditioning and AM251, URB597 or URB597+AM251 on general locomotor behaviours and defecation in formalin-treated rats

Two-way ANOVA revealed no significant main effect of fear-conditioning ($F(1,64) = 0.403$, $p = 0.528$), treatment ($F(3,64) = 0.428$, $p = 0.734$) or fear-conditioning*treatment ($F(3,64) = 0.273$, $p = 0.845$) on the distance moved in the arena over the 30-minute testing period (Table 2.2).

Two-way ANOVA revealed no significant main effect of fear-conditioning ($F(1,64) = 0.296$, $p = 0.588$), treatment ($F(3,64) = 1.317$, $p = 0.277$) or fear-conditioning*treatment ($F(3,64) = 0.638$, $p = 0.594$) on time spent walking in the arena over the 30-minute testing period (Table 2.2).

Two-way ANOVA revealed a significant main effect of fear-conditioning ($F(1,64) = 14.663$, $p < 0.001$), but not treatment ($F(3,64) = 1.955$, $p = 0.130$) or fear-conditioning*treatment ($F(3,64) = 0.110$, $p = 0.954$) on time spent rearing in the arena over the 30-minute testing period.

Further post-hoc analysis revealed no significant differences between the treatment groups (Table 2.2).

Two-way ANOVA revealed a significant main of effect of fear-conditioning ($F(1,64)=5.821$, $p<0.05$) but not treatment ($F(3,64)=1.10$, $p=0.356$) or fear-conditioning*treatment ($F(3,64)=0.256$, $p=0.875$) on time spent grooming in the arena over the 30-minute testing period. Further post-hoc analysis revealed no significant differences between the treatment groups (Table 2.2).

Kruskal Wallis comparisons revealed a significant effect ($\chi^2(8)=34.064$, $p<0.001$) for the amount of defecation in the arena (no. of pellets) over the course of the 30-minute testing period. Further post-hoc analysis by Dunn-Bonferroni revealed no significant differences between the treatment groups (Table 2.2).

Groups	Distance Moved (cm)	Walking (s)	Rearing (s)	Grooming (s)	Defecation (number of pellets)
NFC Vehicle	5555±849	166±16	28±8	67±23	0(4)
NFC AM251	5902±880	167±21	31±7	112±17	0(3)
NFC URB597	4973±1015	144±22	16±5	63±22	0(2)
NFC URB597+AM251	5181±1120	137±27	6±1	57±32	0(4)
FC Vehicle	5756±709	155±19	51±11	107±22	5(4)
FC AM251	4931±770	181±23	64±21	130±23	5(3)
FC URB597	5110±817	186±	47±9	111±19	5(4)
FC URB597+AM251	4184±1056	126±28	38±11	116±35	4(5)

Table 2.2: Effects of fear-conditioning and bilateral microinjection of AM251, URB597 or URB597+AM251 on distance moved, walking, rearing, grooming and defecation in formalin treated rats. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, vs NFC counterparts (Dunn-Bonferroni). All data are expressed as mean \pm SEM except for defecation which is median (interquartile range) (n=7-12 per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

2.3.5 Effect of fear-conditioning and URB597 on AEA, OEA, PEA or 2-AG levels in the ACC of formalin-treated rats

Two-way repeated measures ANOVA revealed no significant main effect of fear-conditioning ($F(1,32)=0.230$, $p=0.632$), treatment ($F(1,32)=0.849$, $p=0.364$) or fear-conditioning*treatment ($F(1,32)=0.279$, $p=0.601$) on the levels of AEA in the ACC (Figure 2.12).

Two-way repeated measures ANOVA revealed no significant main effect of fear-conditioning ($F(1,32)=0.202$, $p=0.657$), treatment ($F(1,32)=1.140$, $p=0.294$) or fear-conditioning*treatment ($F(1,32)=1.625$, $p=0.212$) on the levels 2-AG in the ACC (Figure 2.12).

Two-way repeated measures ANOVA revealed no significant main effect of fear-conditioning ($F(1,32)=0.049$, $p=0.827$), treatment ($F(1,32)=0.521$, $p=0.476$) or fear-conditioning*treatment ($F(1,32)=0.040$, $p=0.842$) on the levels of PEA in the ACC (Figure 2.12).

Two-way repeated measures ANOVA revealed no significant main effect of fear-conditioning ($F(1,32)=0.279$, $p=0.601$), treatment ($F(1,32)=0.147$, $p=0.704$) or fear-conditioning*treatment ($F(1,32)=0.583$, $p=0.451$) on the levels of OEA in the ACC (Figure 2.12).

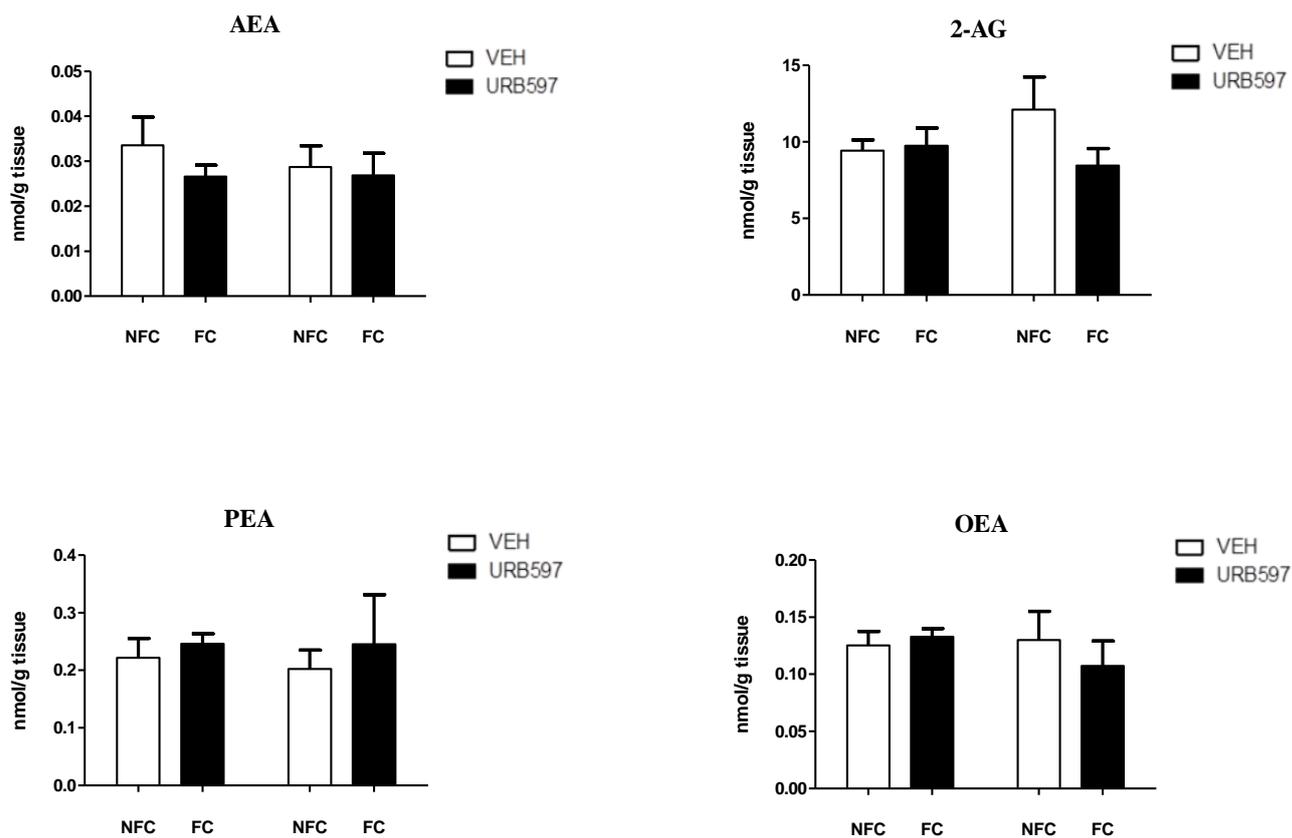


Figure 2.12: Effects of fear-conditioning and bilateral microinjection of URB597 on AEA, 2-AG, PEA or OEA levels in the ACC. All data are expressed as mean \pm SEM (n=7-12 per group); NFC-Non-fear-conditioned; FC-fear-conditioned; VEH-vehicle

2.4 Discussion

The data presented herein suggest that URB597 and AM251 in the rat ACC do not regulate formalin-evoked nociceptive behaviour, conditioned fear in the presence of nociceptive tone or FCA. In the absence of contextual fear-conditioning, administration of the CB₁ receptor antagonist/inverse agonist AM251 or the FAAH inhibitor URB597, alone or in combination, had no effect on formalin-evoked nociceptive behaviour. Re-exposure of VEH-treated rats to a context previously paired with footshock significantly reduced formalin-evoked nociceptive behaviour (i.e. induced FCA) and increased contextually induced freezing and 22kHz ultrasonic vocalisation in the presence of nociceptive tone in FC rats. Intra-ACC administration of AM251 or URB597, alone or in combination had no effect on the expression of FCA or fear-related behaviours. Neither FCA nor intra-ACC administration of URB597 were associated with alterations in the levels of endocannabinoids or related n-acylethanolamines in the ACC at the end of the 30-minute trial. These data suggest that FAAH substrates and CB₁ receptor blockade in the ACC have no effect on the expression of FCA, formalin-evoked nociceptive behaviour or contextually-induced fear in the presence of nociceptive tone. These results also corroborate our previous work (Rea et al., 2018) suggesting that in the mPFC the regulation of the expression of FCA occurs in a subregion-specific manner, with URB597 and AM251 in the ACC, IL and PrL playing distinct and differential modulatory roles in expression of FCA and fear in the presence of nociceptive tone.

In the present study, VEH-treated FC rats exhibited a reduction in formalin-evoked nociceptive behaviour upon re-exposure to a context previously paired with an aversive stimulus (i.e. footshock), confirming the expression of FCA. This finding is consistent with a number of previous studies, within our own group and others, investigating FCA (Fanselow, 1984, Finn et al., 2004, Olango et al., 2012, Rea et al., 2013, Roche et al., 2007, Helmstetter, 1992, Helmstetter and Bellgowan, 1993, Rea et al., 2014a, Rea et al., 2009).

Intra-ACC administration of the FAAH inhibitor URB597 or the CB₁ receptor antagonist AM251 had no effect on formalin-evoked nociceptive behaviour. This is comparable to other studies from our group where URB597 and AM251 had no effect in the IL and PrL on formalin-evoked nociceptive behaviour in the absence of contextual fear (Rea et al., 2018). In contrast to results presented in this chapter, increasing the endogenous tone of AEA supraspinally has been shown to produce antinociceptive effects in rodent models of inflammatory pain (Kaczocha et al., 2014, Roche et al., 2007). Moreover, CB₁ receptor antagonism has been shown to either enhance or inhibit pain in rat models of inflammatory, mechanical and thermal

nociceptive tests (Galdino et al., 2014, Roche et al., 2007). The opposing roles here may be due to the route of administration (systemic vs. intra-BLA) or the nociceptive tests performed (mechanical and thermal vs. inflammatory). A study from our own group indicates that microinjection of PEA into the ACC can reduce inflammatory pain-related behaviour in the first phase of the formalin test, via possible AEA-induced activation of CB₁ receptors (Okine et al., 2016). Moreover, Okine et al., found that AM251 alone induced a significant reduction in formalin-evoked nociceptive behaviour in the first phase of the formalin test (Okine et al., 2016). FAAH has multiple substrates including AEA, PEA and OEA and thus blocking its activity should increase the endogenous tone of these ligands, possibly exerting a behavioural effect. However, the study presented here only looked at the second phase of formalin-evoked inflammatory pain and this may account for the lack of effect of the drugs on nociceptive behaviour in the absence of contextual fear and the absence of any drug induced changes on endocannabinoid levels in the ACC (discussed later). To determine the role of the endocannabinoid system in the mPFC on formalin-evoked nociceptive behaviour, future studies should look at FAAH inhibition and CB₁ receptor agonism and antagonism in the ACC and mPFC across the first and second phases of the formalin test in the absence of contextually induced fear.

Intra-ACC administration of the FAAH inhibitor URB597 also had no effect on the expression of FCA. This result contrasts with previous studies where administration of URB597 systemically or directly into the ventral hippocampus or PrL enhanced FCA, while its administration into the IL attenuated FCA (Finn et al., 2004, Ford et al., 2011, Rea et al., 2018). The results of the present study also revealed that intra-ACC administration of AM251 had no effect on expression of FCA. CB₁ receptor blockade by AM251 or rimonabant have been shown to attenuate FCA when administered systemically or injected directly into the IL, PrL, dlPAG and BLA (Finn et al., 2004, Olango et al., 2012, Olango et al., 2014, Roche et al., 2007) (Rea et al., 2018). The lack of effect of CB₁ receptor blockade in the ACC on FCA highlights region-specific effects of the endocannabinoid system supraspinally on this form of endogenous analgesia. These results are the first to suggest that unlike the ventral hippocampus (Ford et al., 2011), dlPAG (Olango et al., 2012), BLA (Rea et al., 2013, Roche et al., 2007) and the other two regions of the mPFC the IL and PrL (Rea et al., 2018), the ACC does not seem to be an important neural substrate for FAAH substrate/CB₁ receptor-mediated FCA, although other endocannabinoids such as 2-AG acting at other receptors (e.g. 2-AG at CB₂ receptors – see Chapter 5) may play a role. The ACC shares extensive direct and indirect connections with

the aforementioned brain regions (IL, PrL, amygdala and PAG). Despite not having a clear independent role in CB₁ receptor mediated FCA, the ACC is ideally positioned to integrate and modulate the inputs and outputs of these regions as they exert their effects and thus may have an indirect role via these regions in endocannabinoid-mediated FCA.

Focusing on the mPFC, the results from the present study are in contrast with previous studies investigating the role of the endocannabinoid system in the IL and PrL in FCA. In the PrL, CB₁ receptor blockade attenuated the expression of FCA while FAAH inhibition enhanced its expression. In the IL, either CB₁ receptor blockade or FAAH inhibition attenuated the expression of FCA (Rea et al., 2018). However, in the present study, CB₁ receptor blockade and FAAH inhibition in the ACC had no effect on FCA expression suggesting that the endocannabinoid system within the mPFC may regulate FCA in a subregion-specific manner. The three regions of the mPFC are located very close together in the brain. The ACC is reciprocally connected with both the IL and PrL and may play a role in modulating their output (Vertes, 2002). In rodents, the IL and PrL subregions have been shown to differentially affect acquisition, consolidation and expression of contextually conditioned fear (Corcoran and Quirk, 2007, Sharpe and Killcross, 2014, Vidal-Gonzalez et al., 2006, Almada et al., 2015, Sierra-Mercado et al., 2011). As mentioned above URB597 differentially affects the expression of FCA in both the IL and PrL, attenuating and enhancing it respectively. It is possible that injecting a drug such as URB597 into the ACC dually affects neuronal activity in the IL and PrL thus cancelling out any behavioural effects it may have. Further studies using optogenetic manipulation to target specific neuronal cell populations in each of these regions would allow us to better understand the role that each of these subregions play and how they interact in the expression of FCA.

Fear-conditioning significantly increased the duration of the fear-related behaviour in the form of increased duration of freezing and 22kHz ultrasonic vocalisation in the presence of nociceptive tone in VEH-treated rats. Intra-ACC administration of AM251 or URB597, alone or in combination, had no effect on the expression of fear-related behaviours in FC rats. In contrast to the results presented here, systemic or intra-dIPAG CB₁ receptor antagonism has been shown to attenuate fear-related behaviours (freezing and 22kHz ultrasonic vocalisation respectively) in FC rats (Finn et al., 2004, Olango et al., 2014, Olango et al., 2012) while URB597 in the ventral hippocampus increased the duration of freezing in the presence of nociceptive tone in FC rats (Ford et al., 2011). In the mPFC, intra-IL but not intra-PrL administration of AM251 or URB597 attenuates the expression of contextually induced

freezing in the presence of nociceptive tone in FC rats (Rea et al., 2018). These results suggest that FAAH substrates and CB₁ receptors within the mPFC regulate fear-related behaviour, like FCA, in a subregion-specific manner. Future studies should address whether this differential modulation of fear-related behaviours and FCA is achieved via differential modulation of the pathways connecting the mPFC subregions to downstream brain regions involved in pain and fear (e.g. the amygdala and PAG), or more locally within the three subregions themselves via alteration of incoming fear or nociceptive information.

There were no significant changes in the levels of AEA, 2-AG, PEA or OEA in the ACC of rats upon fear-conditioning. This is in line with previous studies suggesting that re-exposure to a context previously paired with footshock had no effect on the levels of AEA or 2-AG in the mPFC (Marsicano et al., 2002). However, AEA and/or 2-AG within the ventral hippocampus (Ford et al., 2011), dorsolateral periaqueductal gray (Olango et al., 2012) or basolateral amygdala (Rea et al., 2013) have been shown to be increased in formalin-treated rats following re-exposure to the same aversive context. Thus, fear conditioning in the context of nociceptive tone differentially modulates endocannabinoid levels in discrete brain regions. A further finding of this study was that URB597 did not increase the levels of endocannabinoids or n-acylethanolamines in the ACC. Similarly, previous studies have shown that URB597 administration (at the same dose or lower) into the IL, PrL or insular cortex did not alter levels of endocannabinoids or n-acylethanolamines (McGowan, 2015, Sticht et al., 2016). However, dose-dependent increases in AEA were observed upon intra-vIPAG administration of URB597 (Maione et al., 2006), although the doses used in this study were much higher (between 2.5mM and 20mM) than those used in this chapter (0.1mM). Moreover, previous work by Hohmann et al., has found a significant increase in AEA following URB597 treatment at a lower dose (1µM) than that used in this chapter in rat brain slices (Hohmann et al., 2005). Endocannabinoids are synthesised on demand and are rapidly degraded (Muccioli, 2010), this may have contributed to the lack of significant changes exhibited between FC and NFC VEH treated rats. The present study measured tissue levels rather than extracellular levels of endocannabinoids and n-acylethanolamines which may have contributed to the lack of significant changes seen in URB597 treated rats. The levels of the endocannabinoids were measured 45 minutes post URB597 administration. URB597 may have increased the levels of AEA, PEA and OEA at an earlier time point but the levels may have returned to baseline levels by the time they were quantified. As mentioned previously, fast green dye is injected post-mortem to clearly visualise cannula placement and drug diffusion. The ACC is a large region

spanning from Bregma 3.7mm to -0.26mm. Punch dissection of excessive tissue rather than just that of the dye spread may have ‘diluted’ between-group differences within the ACC making them more difficult to detect. Future studies should punch only where drug diffusion occurs to allow for accurate detection of drug effects.

Overall, the data suggest that FAAH substrates in the ACC do not play a direct role in the expression of FCA and fear-related behaviours in the presence of nociceptive tone. Behaviourally, URB597 had no effect on the expression of fear-related behaviours or FCA. The mass spectrometry data revealed that in the ACC, the expression of these behaviours, in FC rats compared to NFC rats, was not associated with any significant FAAH substrate alterations (AEA, PEA and OEA). Similarly, URB597 had no effect on FAAH substrate levels in either FC or NFC rats. If the expression of FCA and associated fear-related behaviours in the presence of nociceptive tone were dependent on FAAH substrates then an associated alteration in AEA, PEA or OEA could be expected in the ACC of rats, an effect that would be modulated with a FAAH inhibitor. Taking all of this into account, as we did not see any behavioural or tissue level changes it is possible that FAAH substrates in the ACC do not play a direct role in the expression of FCA and fear in the presence of pain.

In conclusion, the present study provides new evidence to suggest that FAAH substrates and CB₁ receptors in the rat ACC do not seem to play a role in contextually-induced fear in the presence of nociceptive tone or FCA. Further work is required to determine their role in formalin-evoked nociceptive behaviour across both phases of the formalin test. Furthermore, our data, when taken together with previous data generated in our laboratory (Rea et al., 2018), suggest that endocannabinoid-mediated regulation of these behaviours occurs in a mPFC subregion-specific manner. 2-AG may represent a new and interesting component of the endocannabinoid system to target pain and anxiety disorders and the role of 2-AG and MAGL inhibition in the ACC, IL and PrL in formalin-evoked nociceptive behaviour, contextually induced fear in the presence of nociceptive tone or FCA remain to be investigated. This will be studied in the following chapters (chapters 3, 4 and 5). Elucidation of the role of the endocannabinoid system in different subregions of the mPFC in fear-pain interactions may facilitate increased understanding of, and development of new therapeutic approaches for, pain- and fear-related disorders and their comorbidity.

Chapter 3 : The role of 2-AG in the infralimbic cortex in formalin-evoked nociceptive behaviour, fear-conditioned analgesia and conditioned fear in the presence of nociceptive tone in rats

3.1 Introduction

The IL is a subregion of the mPFC believed to be homologous with Brodmann's area 25 in the human brain (Ongur and Price 2000). The IL has numerous projections, including but not limited to other subregions of the mPFC (PrL and ACC), orbital, insular, entorhinal and piriform cortices, olfactory nucleus and forebrain, bed nucleus of the stria terminalis, substantia nigra, nucleus accumbens shell, hypothalamus, parabrachial nucleus, the medial, basomedial, cortical and central nuclei of the amygdala, thalamus, hippocampus and PAG (Vertes, 2004). Within the mPFC the IL projections are more widespread than those of the PrL. The large number of projections of the IL reflect its numerous functions including, regulation of blood pressure and the cardiovascular system, reward and punishment, pain modulation, anxiety, responses to stress, and fear extinction (Vertes, 2004).

The IL has an important role in fear regulation, mediating extinction and fear suppression (Giustino and Maren, 2015). An early lesion study in rats suggested a role for the IL in the consolidation of extinction memories (Quirk et al., 2000). Electrophysiological methods *in vivo* in rats then provided evidence that conditioned stimulus (CS)-evoked responses in the IL correlate with successful extinction recall following fear-conditioning with electrical stimulation (Milad and Quirk, 2002, Milad et al., 2004). Similarly, multichannel unit recording in rats found that high-frequency IL bursting is required for successful extinction (Burgos-Robles et al., 2007) while this high-frequency bursting is diminished or impaired under conditions where immediate extinction fails (Chang et al., 2010). Microstimulation of the IL has also been shown to inhibit conditioned fear following extinction 24 hours post conditioning with auditory tone and electrical stimulation in rats (Vidal-Gonzalez et al., 2006). In agreement with this, inactivation of the IL impaired recall of extinction and within-session extinction in rats (Sierra-Mercado et al., 2006, Sierra-Mercado et al., 2011, Laurent and Westbrook, 2009). In humans, the IL has been suggested to regulate fear suppression and extinction (Phelps et al., 2004) and its projections may be dysregulated in patients exhibiting PTSD (Gilboa et al., 2004, Garfinkel et al., 2014). The above information, paired with the fact that the IL projects to areas

also involved in fear-related and extinction behaviour (amygdala and hippocampus (Vertes, 2004)), suggests a role for IL activity in the top-down modulation of fear-related processes.

Unlike its role in fear-related behaviour, the role of the IL in pain is not as well characterised. In healthy rats, David-Pereira et al., found that glutamate-induced activation of the IL resulted in a slow onset pronociceptive effect in the heat-evoked paw withdrawal test while inhibition of the IL via local anaesthesia also facilitated nociception in healthy rats (David-Pereira et al., 2016). In contrast to this study, Jiang et al., found that inactivation of the rat IL had no effect on the acquisition and expression of formalin-conditioned place avoidance, suggesting that the modality of pain (heat and arthritis vs. formalin inflammation) may contribute to IL recruitment (Jiang et al., 2014). Intra-IL administration of ethanol lowered the mechanical pain threshold in the Von Frey test in rats without having any effect on thermal pain sensitivity, an effect mediated by GABA_A receptors (Geng et al., 2016). A bilateral increase in microglial density has been shown in the IL of rats, but not mice, following spared nerve injury, an effect not seen in the ACC or PrL (Chu Sin Chung et al., 2017). A decrease in BDNF has been seen in the IL but not PrL of rats following CFA-induced inflammatory pain (Yue et al., 2017). As previously mentioned, the IL projects to regions with well characterised pain functions such as the amygdala and PAG (Vertes, 2004). These projections, together with the above studies citing a clear function of the IL in pain responding, suggest a possible role for the IL in the top-down modulation of pain.

A previous study from our laboratory suggested a role for the PFC in pain, fear and endogenous pain suppression. Butler et al., found that rats expressing either formalin-evoked nociceptive behaviour or conditioned fear-related behaviour exhibited increased MAPK activity in the PFC, an effect not observed in rats expressing FCA (Butler et al., 2011). More recent work in our laboratory has highlighted a role for the endocannabinoid system, in particular FAAH and CB₁, in the rat mPFC in FCA. The FAAH inhibitor URB597 had opposing effects in the IL and PrL (Rea et al., 2018) attenuating and enhancing FCA respectively, while having no effect in the ACC (chapter 2). The CB₁ receptor antagonist AM251 in contrast attenuated FCA in the IL and PrL while having no effect in the ACC (Rea et al., 2018 and Chapter 2).

2-AG represents a new and interesting component of the endocannabinoid system to target and treat both pain and anxiety disorders. Converging data have demonstrated a key role for 2-AG in the regulation of fear, anxiety and pain (Bluett et al., 2017, Busquets-Garcia et al., 2011, Sciolino et al., 2011, Sumislowski et al., 2011, Rea et al., 2014a, Wilkerson et al., 2017,

Llorente-Berzal et al., 2015, Hartley et al., 2016), see the discussion for further detail. In the ventral hippocampus 2-AG administration significantly reduced contextually induced freezing in saline-treated rats, and reduced formalin-evoked nociceptive behaviour in NFC rats. In contrast, 2-AG microinjection had no effect on fear responding in formalin-treated rats, and no effect on FCA. The fear-related effects of 2-AG were blocked upon co-administration with a CB₁ antagonist (Rea et al., 2014a). Moreover, MAGL inhibition enhanced the expression of SIA in the PAG of rats (Hohmann et al., 2005). However, no studies to date have investigated the role of 2-AG and MAGL inhibition in the IL in fear-pain interactions.

This chapter will assess the role of MAGL inhibition in the IL in formalin-evoked nociceptive behaviour, conditioned fear in the presence of nociceptive tone and FCA with the following hypothesis: MAGL inhibition in the IL will attenuate the expression of FCA and conditioned fear in the presence of nociceptive tone while having no effect on formalin-evoked nociceptive behaviour. Therefore, the overall aims of the study described in this chapter were:

- To determine the role of 2-AG in the IL in formalin-evoked nociceptive behaviour, expression of fear behaviour in the presence of formalin-evoked nociceptive tone and the expression of FCA. This was achieved by examining the effects of intra-IL administration of the MAGL inhibitor, MJN110 alone or in combination with the CB₁ receptor antagonist/inverse agonist, AM251, on formalin-induced nociceptive behaviour, fear-related behaviour in the presence of nociceptive tone and FCA in rats.
- To examine if behavioural changes are associated with alterations in endocannabinoid levels in the IL and associated mPFC regions using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

3.2 Materials and Methods

3.2.1 Animals:

Male Lister-Hooded rats (260–325g at time of behavioural testing); Charles River, Margate, Kent, UK) were used. Before surgery, animals were housed 2-3 per flat bottomed cage (L: 45 x H: 20 x W: 20cm) containing wood shavings as bedding (LBS, Surrey, UK). Animals were kept at a constant temperature ($21^{\circ}\text{C} \pm 2^{\circ}\text{C}$) under standard lighting conditions (12:12h light–dark, lights on from 0700 to 1900h). Experiments were carried out during the light phase between 0800 and 1700h. Food (14% Harlan-Teklad-2014 Maintenance Diet, Harlan Laboratories, Belton, Loughborough, UK) and water were provided *ad libitum*. The experimental protocol was carried out after approval by the Animal Care and Research Ethics Committee, National University of Ireland Galway, under license from the Health Products Regulatory Authority and in compliance with the European Communities Council directives 86/609 and 2010/63 and conformed to the ARRIVE guidelines.

3.2.2 Cannulae implantation:

See Chapter 2 section 2.2.2 for details on cannulae implantation via stereotaxic surgery.

Briefly, stainless steel guide cannulae (9mm length, 22G, Bilaney Consultants, Sevenoaks, UK) were stereotaxically implanted bilaterally 1mm above the right and left IL (AP + 2mm relative to bregma, ML \pm 1.5mm relative to bregma and at a 12° angle, DV – 3.6mm from dura, toothbar set at -3mm).

Following surgery, animals were singly housed and a single daily dose of enrofloxacin (2.5mg/kg, s.c., Baytril; Bayer Ltd., Dublin, Ireland) was administered for the following four days. A minimum of 6 days was allotted to allow rats to recover before any experimentation commenced. During this period, the rats were handled, stylets checked, and their body weight and general health monitored.

3.2.3 Chemicals and drug preparation:

Formalin (37% formaldehyde solution), DMSO (dimethyl sulfoxide, 100%), AM251 (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) and MJN110 (2,5-dioxopyrrolidin-1-yl 4-(bis(4-

chlorophenyl)methyl)piperazine-1-carboxylate) were purchased from Sigma-Aldrich, Dublin, Ireland.

On test days, solutions of 2µg/µl MJN110 (MAGL inhibitor), or 2µg/µl MJN110 + 2mM AM251 (CB₁ receptor antagonist/inverse agonist) were prepared using stock solutions and 100% DMSO vehicle. A solution of 2.5% formalin (Sigma-Aldrich, Dublin, Ireland) was prepared from a 37% stock solution diluted with 0.9% sterile saline.

These doses of AM251 and MJN110 are based on previous work carried out by our laboratory and evidence from the literature which indicated *in vivo* efficiency in rodent models (Lisboa et al., 2010, Freitas et al., 2013, Rea et al., 2018, Wills et al., 2016, Sticht et al., 2016).

3.2.4 Experimental procedure:

The experimental procedure was identical to that described in chapter 2 section 2.2.4.

On test days, rats received intra-IL microinjection (0.3µl per side) of either vehicle (VEH; 100% DMSO), the MAGL inhibitor MJN110 (2µg/µl) or the CB₁ receptor antagonist MJN110+AM251 (MJN110: 2µg/µl, AM251:2mM). A group receiving AM251 alone was not included because the effects of intra-IL administration of AM251 alone on the same behavioural parameters in an identical experimental design were investigated by Rea et al., 2018.

After 30-minutes in the arena, rats were removed and euthanised by decapitation. Brains and spinal cords were rapidly removed within a 2-minute timeframe, snap-frozen on dry ice and stored at -80°C. After decapitation, 0.3µl of 2% fast green dye (dissolved in DMSO) was also injected into the left and right IL for post-mortem confirmation of microinjection sites during cryo-sectioning.

This design resulted in 6 experimental groups (Starting n=12 per group for surgery; final n=9-11 per group for data analysis) as illustrated in Table 3.1.

Group	Conditioning	Formalin i.pl.	Drug/Vehicle	N
1	FC	Formalin	100% DMSO	9
2	NFC	Formalin	100% DMSO	9
3	FC	Formalin	MJN110	11
4	NFC	Formalin	MJN110	9
5	FC	Formalin	MJN110+AM251	11
6	NFC	Formalin	MJN110+AM251	11

Table 3.1: Summary of experimental groups: i.pl., intraplantar; FC, fear-conditioned; NFC, non – fear-conditioned

3.2.5 Brain removal:

See chapter 2 section 2.2.5.

3.2.6 Behavioural analysis:

See chapter 2 section 2.2.6.

3.2.7 Histological verification of intracerebral injection sites:

See chapter 2 section 2.2.7.

3.2.8 Tissue isolation by Palkovits punch:

Frozen coronal brain sections were collected at 150 μ m on a cryostat at -20°C. Slices were collected on superfrost glass slides and microinjection site positions were determined and noted on photocopied images from rat brain atlas (Paxinos and Watson, 1998) prior to data analysis. Regions of interest in the mPFC were punched from the frozen 150 μ m coronal sections using cylindrical brain punchers (Harvard Apparatus, Massachusetts, USA; internal diameters 0.5 and 2mm). Tissue from the left and right IL (Bregma 3.20 to 2.20mm, 0.5mm puncher); PrL (Bregma 3.70 to 2.20mm, 0.5mm puncher); and ACC (Bregma 3.70 to 2.20mm, 0.5mm puncher and bregma 1.70 to -0.26mm, 2mm puncher), was punched. Punched regions were kept frozen throughout the collection procedure. After collection, punches were placed in

labelled 1.5ml microfuge tubes (Sarstedt, Nümbrecht, Germany), tissue weight recorded, and stored at -80°C.

3.2.9 Measurement of levels of endocannabinoids in the medial prefrontal cortex:

See chapter 2 section 2.2.9.

Amendments to protocol:

- Tissue levels were measured from the IL, PrL and ACC.
- Before tissue homogenisation, 75µl of acetonitrile was added to tissue samples to allow for equal volume in both samples and standard curves.
- Samples and standards were not evaporated or resuspended in 40µl 65% acetonitrile before running.

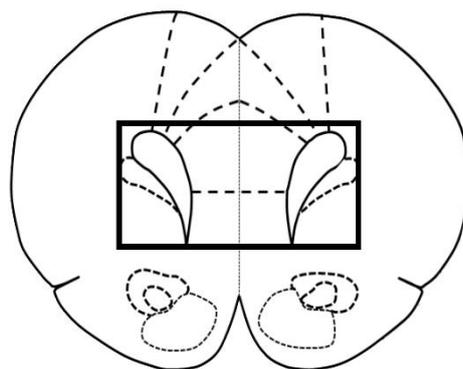
3.2.10 Statistical analysis:

The IBM SPSS statistical software package (SPSS v23.0 for Microsoft Windows; Chicago, IL, USA) was used to analyse all data. Normality and homogeneity of variance were assessed using Shapiro-Wilk's and Levene's test, respectively. All data except the duration of freezing, 22kHz ultrasonic vocalisation and defecation were found to be parametric. Behavioural data and mass spectrometry data were analysed by 2-way ANOVA, with fear-conditioning and drug treatment as factors. Time course behavioural data were analysed by 2-way repeated measures ANOVA with time as the within-subjects factor and fear-conditioning and drug treatment as the between-subjects factors. Sphericity was tested using Mauchly's Test for Sphericity. If sphericity was violated a Greenhouse-Geisser correction was used. Post-hoc pairwise comparisons were made with Tukey's test when appropriate. Non-parametric data was analysed using Kruskal Wallis analysis of variance and post-hoc analysis performed using Dunns-Bonferroni pairwise comparisons when appropriate. For repeated measures non-parametric data Kruskal Wallis was performed followed by Dunn-Bonferroni post-hoc where appropriate. Data were considered significant when $p < 0.05$. Results are expressed as group means \pm SEM or median with interquartile.

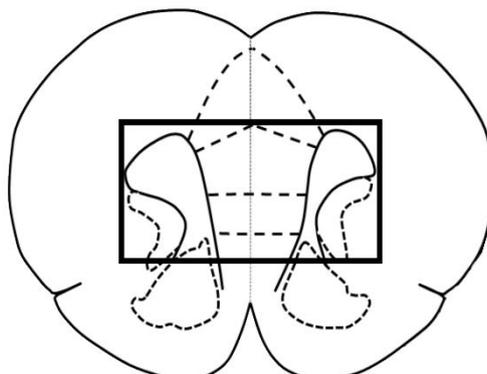
3.3 Results

3.3.1 Histological verification of microinjection sites

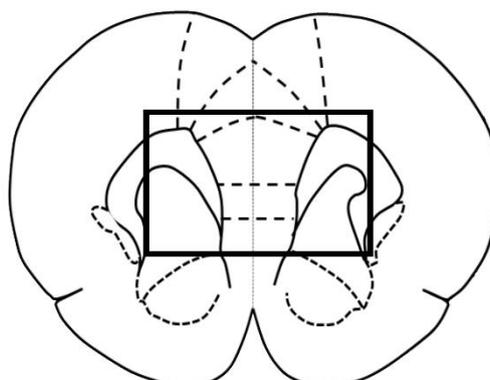
88% of the injections were within the borders of the IL (Figure 3.1) with the remaining 12% placed just lateral to the IL in the corpus callosum or above the IL in the PrL. The data analysed were derived only from rats in which bilateral injections were correctly positioned in the IL.



+3.2 mm from Bregma



+2.7 mm from Bregma



+2.2 mm from Bregma

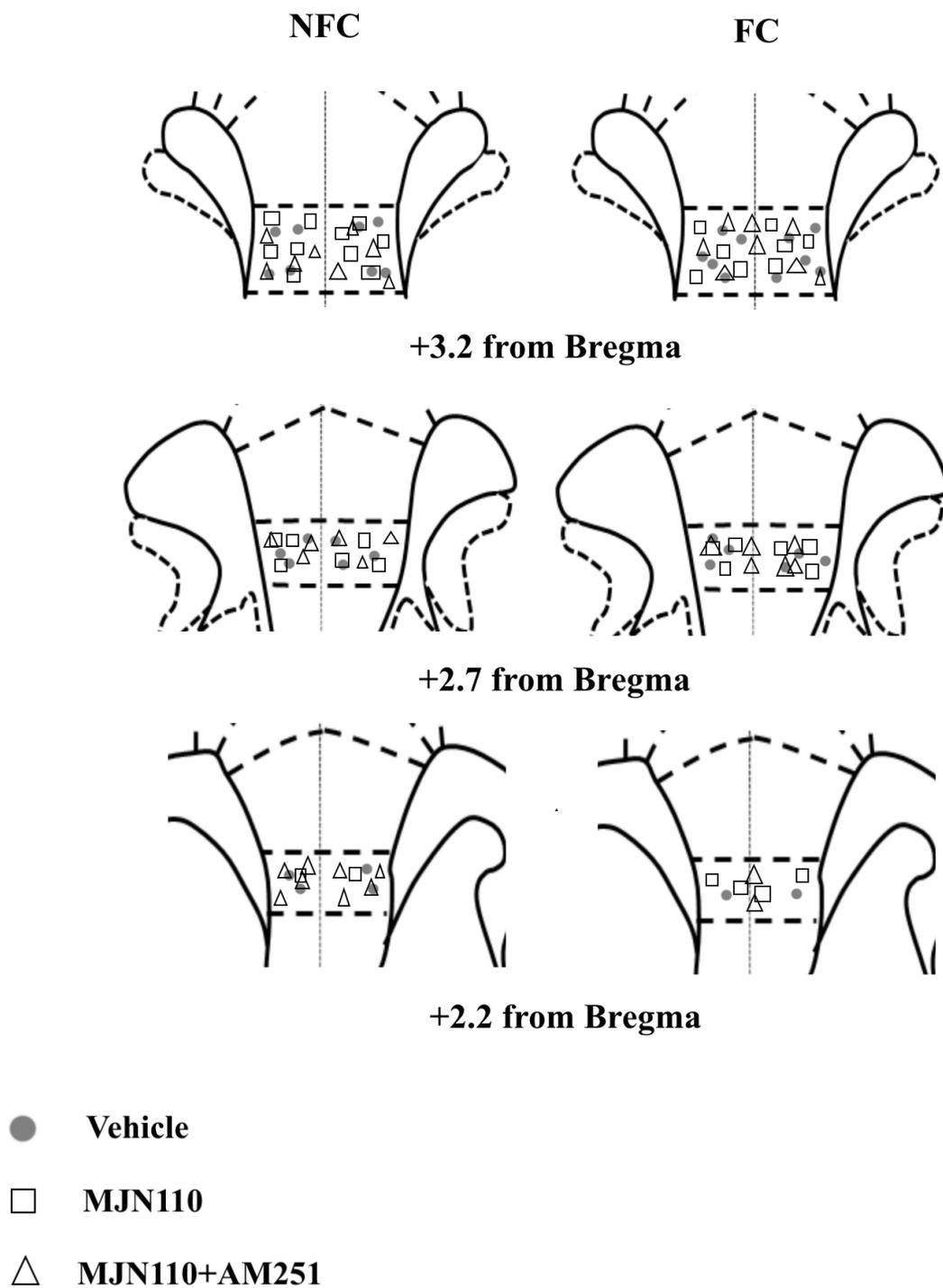


Figure 3.1: Histological verification of injector site location. Adapted from Paxinos & Watson (1998). NFC: Non-fear-conditioned; FC: Fear-conditioned.

3.3.2 Effects of intra-IL administration of MJN110 or MJN110+AM251 on formalin-evoked nociceptive behaviour and FCA

Intra-plantar injection of formalin induced a robust nociceptive response in the form of elevation, licking, biting and shaking of the injected right hind-paw. Two-way ANOVA revealed no significant main effect of fear-conditioning ($F(1,59)=1.433$, $p=0.236$), treatment ($F(2,59)=2.719$, $p=0.075$) or fear-conditioning*treatment ($F(2,59)=0.903$, $p=0.411$) on the change in hind paw diameter pre- versus post-formalin injection (Figure 3.2).

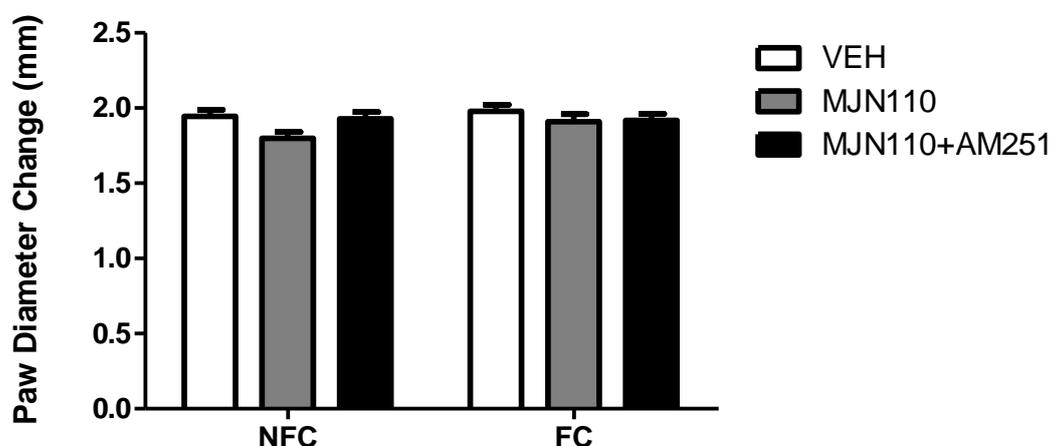


Figure 3.2: Effects of fear-conditioning and bilateral administration of MJN110 or MJN110+AM251 directly into the IL on paw diameter change (mm) pre- and post-formalin injection (oedema). All data are expressed as mean \pm SEM ($n=9-11$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Two-way ANOVA revealed a significant main effect of fear-conditioning ($F(1,54)=58.409$, $p<0.001$), and fear-conditioning*treatment ($F(2,54)=6.144$, $p<0.01$) but not treatment alone ($F(2,54)=2.943$, $p=0.061$) on CPS over the entire 30-minute testing period. Re-exposure of rats to the arena previously paired with footshock resulted in a significant reduction in formalin-evoked nociceptive behaviour, confirming the expression of FCA (FC VEH vs. NFC VEH, $p<0.001$). Intra-IL administration of MJN110+AM251 significantly attenuated the expression of FCA over the course of the 30-minute trial (FC VEH vs. FC MJN110+AM251, $p<0.01$). While MJN110 treated rats are still expressing FCA, there is a trend for this drug to attenuate

FCA (FC MJN110 vs. NFC MJN110 $p < 0.001$). There were no effects of any of the drug treatments in NFC rats (Figure 3.3).

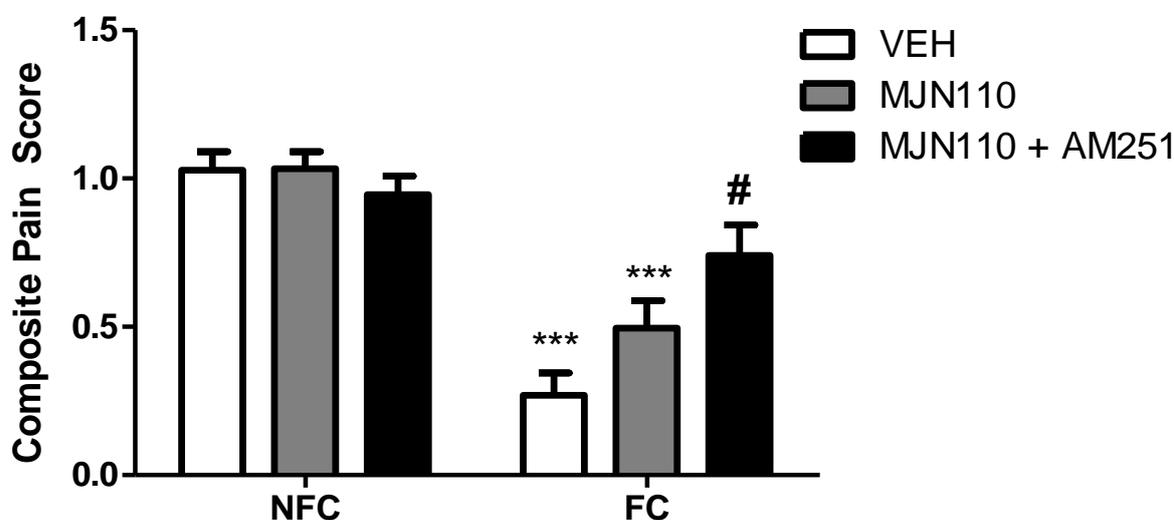


Figure 3.3: Effects of fear-conditioning and bilateral administration of MJN110 and MJN110+AM251 directly into the IL on formalin-evoked nociceptive behaviour in rats over the full 30-minute testing period. *** $p < 0.001$ FC vs. NFC; # $p < 0.01$ vs. FC VEH (Tukey's). All data are expressed as mean \pm SEM (n=9-11 per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Temporal analysis of the data subdivided into 10-minute time bins and using 2-way repeated measures ANOVA revealed a significant main effect of fear-conditioning ($F(1,54)=58.409$, $p < 0.001$) and fear-conditioning*treatment ($F(2,54)=6.144$, $p < 0.01$) but not treatment ($F(2,54)=2.943$, $p=0.061$). Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated ($\chi^2(2)=7.005$, $p < 0.05$) and therefore a Greenhouse-Geisser correction was used. There was a significant main effect of time ($F(1.78,96.10)=47.137$, $p < 0.001$) and time*fear-conditioning ($F(1.78,96.10)=19.037$, $p < 0.001$) but not time*treatment ($F(3.56,96.10)=0.093$, $p=0.978$) or time*fear-conditioning*treatment ($F(3.56,96.10)=1.418$, $p=0.238$) on CPS. Further post-hoc analysis revealed a significant reduction in formalin-evoked nociceptive behaviour in FC VEH treated rats for the entire 30-minute testing period (FC VEH vs. NFC VEH, 0-10mins, 10-20mins, 20-30mins, $p < 0.001$) confirming the expression of FCA throughout the trial. Intra-IL administration of MJN110, alone or in combination with AM251, significantly attenuated the expression of FCA, in the final 10-

minutes of the trial (FC VEH vs. FC MJN110 or FC MJN110+AM251, 20-30mins, $p < 0.05$), while MJN110+AM251 treated rats do not express FCA in the first 20 minutes of the trials, with similar trends for MJN110 treated rats. There were no effects of these drug treatments in NFC animals (Figure 3.4).

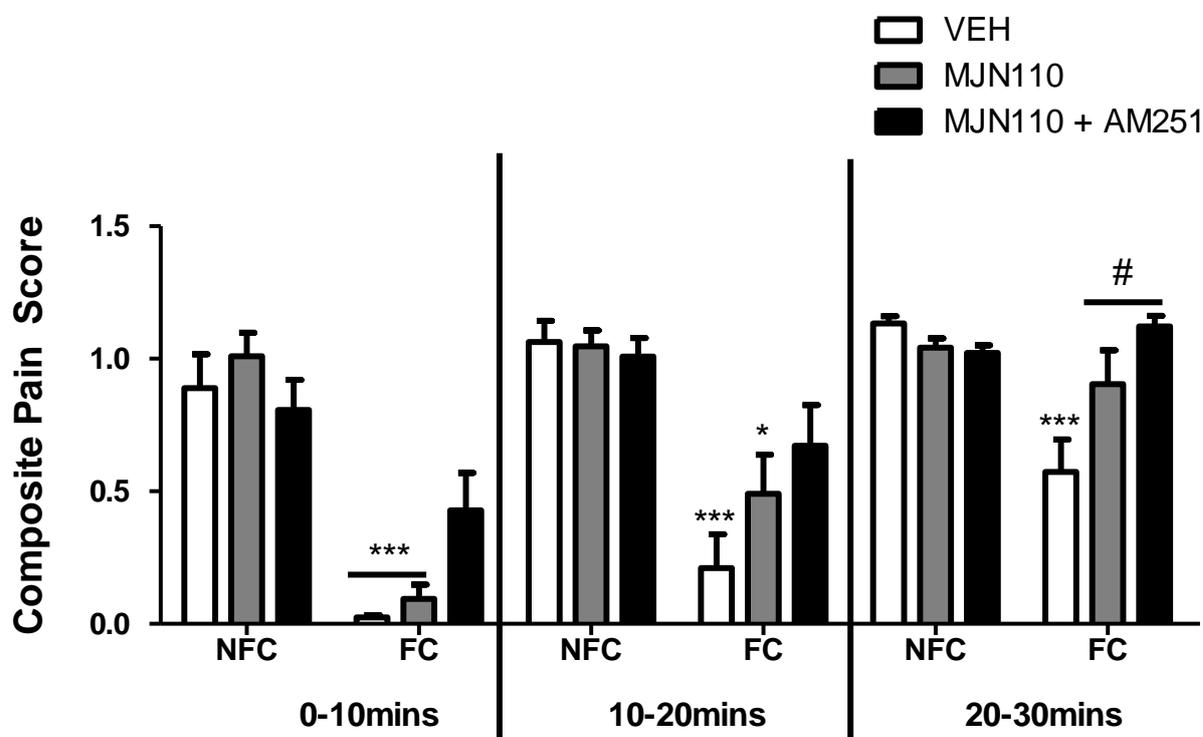


Figure 3.4: Effects of fear-conditioning and bilateral administration of MJN110 and MJN110+AM251 directly into the IL on formalin-evoked nociceptive behaviour in rats over the full 30-minute testing period subdivided into 10-minute time bins. * $p < 0.05$, *** $p < 0.001$ FC vs. NFC; # $p < 0.05$ vs FC VEH (Tukey's). All data are expressed as mean \pm SEM ($n = 9-11$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

3.3.3 Effects of intra-IL administration of MJN110 or MJN110+AM251 on expression of conditioned fear behaviour in the presence of formalin-evoked nociceptive tone

Freezing Behaviour:

Kruskal Wallis comparisons revealed a significant main effect on the duration of freezing ($\chi^2(5) = 48.756$, $p < 0.001$) over the course of the 30-minute testing period. Further post-hoc analysis using Dunn-Bonferroni revealed that FC rats displayed significantly more freezing

(FC VEH vs. NFC VEH, $p < 0.001$) compared to NFC rats over the course of the 30-minute testing period. Neither MJN110 nor a combination of MJN110+AM251 had any effect on the duration of freezing (Figure 3.5).

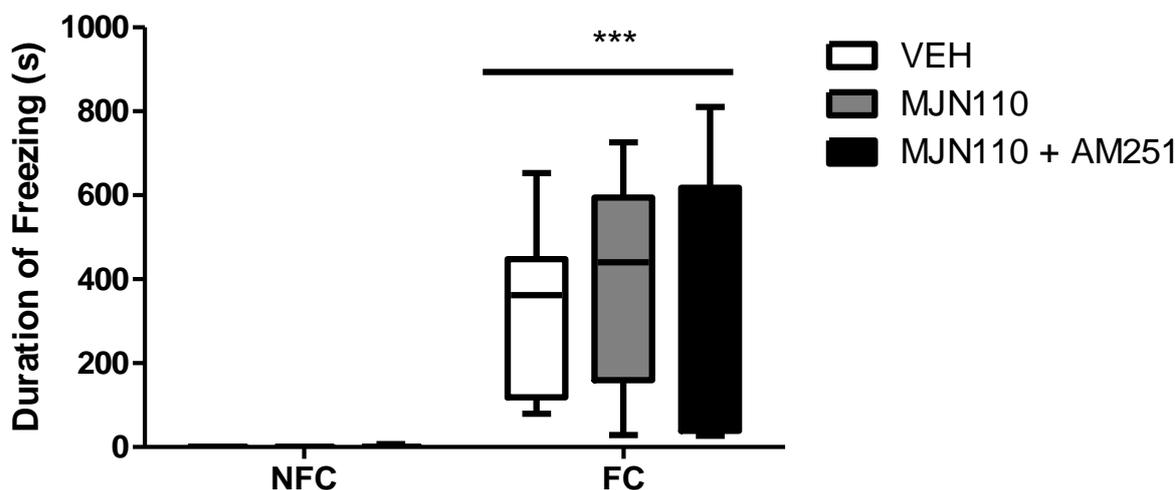


Figure 3.5: Effects of fear-conditioning and bilateral administration of MJN110 and MJN110+AM251 directly into the IL on the duration of freezing in rats over the full 30-minute testing period. *** $p < 0.001$ FC vs. NFC; (Dunn-Bonferroni). All data are expressed as median with interquartile range ($n = 9-11$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Analysis of the data subdivided into 10-minute time bins using Kruskal-Wallis revealed a significant between-group effect on the expression of freezing at 0-10mins ($\chi^2(5) = 50.947$, $p < 0.001$), 10-20mins ($\chi^2(5) = 26.141$, $p < 0.001$) but not 20-30mins ($\chi^2(5) = 7.337$, $p = 0.197$). Post-hoc analysis using the Dunn-Bonferroni test revealed that fear-conditioned rats receiving intra-IL VEH displayed significantly increased freezing behaviour in the first 10-minutes of the trial, compared with NFC VEH treated counterparts (FC VEH vs. NFC VEH; 0-10mins, $p < 0.001$). Neither MJN110 nor a combination of MJN110+AM251 had any effect on the duration of freezing (Figure 3.6).

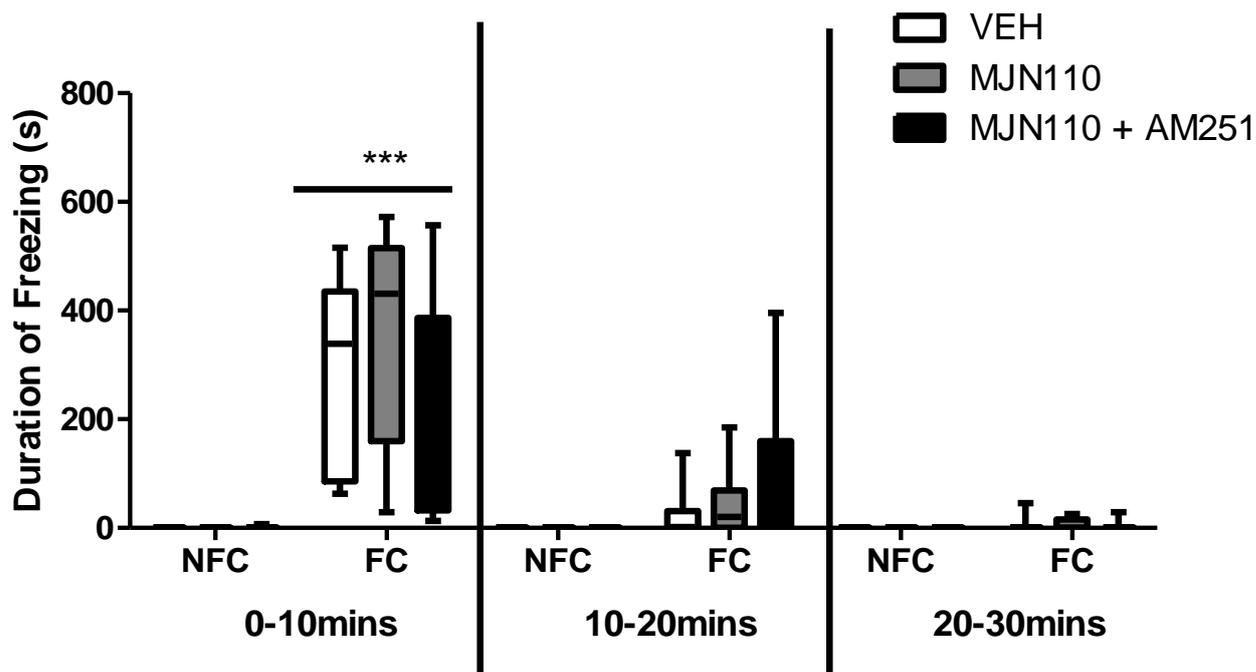


Figure 3.6: Effects of fear-conditioning and bilateral administration of MJN110 and MJN110+AM251 directly into the IL on the duration of freezing in rats over the full 30-minute testing period subdivided into 10-minute time bins. *** $p < 0.001$ FC vs. NFC; (Dunn-Bonferroni). All data are expressed as median with interquartile range ($n = 9-11$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Ultrasonic Vocalisation:

Kruskal Wallis comparisons revealed a significant main effect on the duration of 22kHz ultrasonic vocalization ($\chi^2(5) = 33.755$, $p < 0.001$) over the course of the 30-minute testing period. Further post-hoc analysis using Dunn-Bonferroni revealed that fear-conditioned rats expressed a significantly higher duration of 22kHz ultrasonic vocalization (FC VEH vs. NFC VEH, $p < 0.01$) compared to non-fear conditioned counterparts over the course of the 30-minute testing period. Neither MJN110 nor a combination of MJN110+AM251 had any effect on the duration of ultrasonic vocalisation (Figure 3.7).

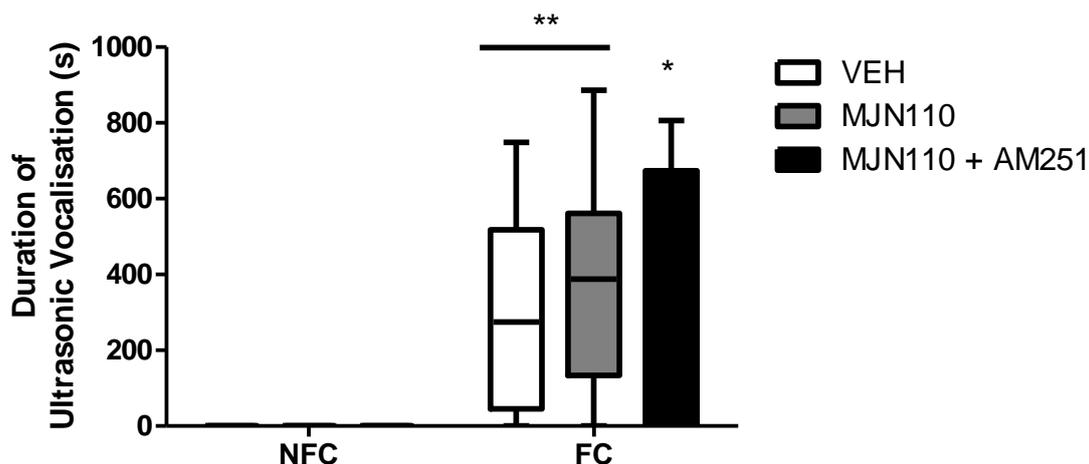


Figure 3.7: Effects of fear-conditioning and bilateral administration of MJN110 and MJN110+AM251 directly into the IL on duration of ultrasonic vocalisation in rats over the full 30-minute testing period. * $p < 0.05$, ** $p < 0.01$ vs. NFC, (Dunn-Bonferroni). All data are expressed as median with interquartile range ($n = 9-11$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Analysis of the data subdivided into 10-minute time bins using Kruskal-Wallis revealed a significant between-group effect on the expression of 22kHz ultrasonic vocalisation at 0-10mins ($\chi^2(5) = 33.765$, $p < 0.001$), 10-20mins ($\chi^2(5) = 20.388$, $p < 0.001$) but not 20-30mins ($\chi^2(5) = 7.585$, $p = 0.181$). Post-hoc analysis using the Dunn-Bonferroni test revealed that fear-conditioned rats receiving intra-IL VEH displayed significantly increased duration of 22kHz ultrasonic vocalisation, compared with NFC VEH treated counterparts for the first 10-minutes of the testing period (FC VEH vs. NFC VEH, 0-10mins, $p < 0.001$). Neither MJN110 nor a combination of MJN110+AM251 had any effect on the duration of ultrasonic vocalisation (Figure 3.8).

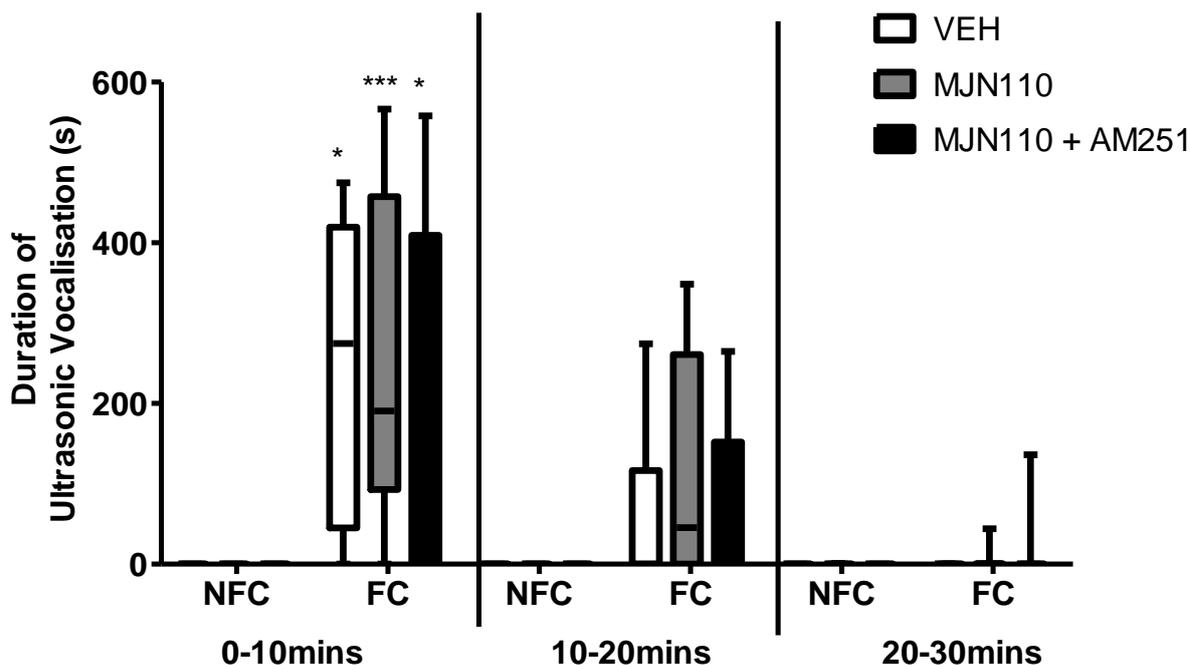


Figure 3.8: Effects of fear-conditioning and bilateral administration of MJN110 and MJN110+AM251 directly into the IL on duration of ultrasonic vocalisation in rats over the full 30-minute testing period subdivided into 10-minute time bins. * $p < 0.05$, *** $p < 0.001$ (Dunn-Bonferroni). All data are expressed as median with interquartile range ($n = 9-11$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

3.3.4 Effects of fear-conditioning and MJN110 or MJN110+AM251 treatment on locomotor behaviours and defecation in formalin-treated rats

Two-way ANOVA revealed a significant main effect of treatment ($F(2,54) = 11.623$, $p < 0.001$) but not fear-conditioning ($F(1,54) = 1.744$, $p = 0.192$) or fear-conditioning*treatment ($F(2,54) = 0.054$, $p = 0.948$) on distance moved (cm) over the course of the 30-minute testing period. Post-hoc analysis revealed treatment with both MJN110 alone and in combination with AM251 significantly decreased distance moved in FC rats (FC VEH vs FC MJN110 and FC MJN110+AM251; $p < 0.05$) (Table 3.2).

Two-way ANOVA revealed a significant effect of fear-conditioning ($F(1,54) = 5.313$, $p < 0.05$) on the duration of walking over the 30-minute trial but not treatment ($F(2,54) = 1.854$, $p = 0.166$) or fear-conditioning*treatment ($F(2,54) = 1.220$, $p = 0.303$). Post-hoc analysis revealed no significant effects (Table 3.2).

Two-way ANOVA revealed a significant effect of fear-conditioning ($F(1,54)=30.950$, $p=0.000$), treatment ($F(2,54)=6.676$, $p<0.01$) and fear-conditioning*treatment ($F(2,54)=4.765$, $p<0.05$) on the duration of rearing. Post-hoc analysis revealed a significant increase in rearing in FC VEH rats compared to their NFC counterparts (FC VEH vs. NFC VEH $p<0.001$) an effect attenuated by treatment with either MJN110 alone or in combination with AM251 (FC VEH vs. FC MJN110, $p<0.01$ or FC MJN110+AM251, $p<0.001$) (Table 3.2).

Two-way ANOVA revealed a significant effect of fear-conditioning ($F(1,54)=6.157$, $p<0.05$) but not treatment ($F(2,54)=1.572$, $p=0.217$) or fear-conditioning*treatment ($F(2,54)=2.264$, $p=0.114$) on the duration of grooming. Post-hoc analysis revealed no significant effects on the duration of grooming over the course of the 30-minute testing period (Table 3.2).

Kruskal Wallis comparisons revealed a significant effect ($\chi^2(5)=35.770$, $p<0.001$) for the amount of defecation in the arena (no. of pellets) over the course of the 30-minute testing period. Further post-hoc analysis by Dunn-Bonferroni revealed that FC rats (FC VEH vs. NFC VEH, $p<0.01$) exhibited a significant increase in defecation over the course of the 30-minute testing period compared to their NFC counterparts. Treatment with MJN110 alone or in combination with AM251 had no effect on the amount of defecation exhibited by rats (Table 3.2).

Treatment Group	Distance Moved (cm)	Walking (s)	Rearing (s)	Grooming (s)	Defecation (number of pellets)
NFC VEH	2038±100	60±13	13±6	60±20	2(2)
NFC MJN110	1485±129	37±8	2±1	26±7	3(2)
NFC MJN110+AM251	1524±143	56±10	9±4	49±15	2(2)
FC VEH	2233±110	92±15	115±20***	93±21	7(3)**
FC MJN110	1608±16#	72±15	44±11#	91±18	6(3)**
FC MJN110+AM251	1638±12#	57±9	36±18#	48±11	7(3)*

Table 3.2: Effects of fear-conditioning and MJN110 or MJN110+AM251 on distance moved, walking, rearing, grooming and defecation in formalin treated rats. All data are expressed as mean \pm SEM except for defecation which is median (interquartile range) (n=9-11 per group). *p<0.05, **p<0.01, ***p<0.001 vs. NFC; #p<0.05 vs FC VEH. FC, fear-conditioned; NFC, non-fear-conditioned; VEH, vehicle.

3.3.5 Effect of fear-conditioning and vehicle or MJN110 on AEA, OEA, PEA or 2-AG levels in the mPFC of formalin-treated rats

Infralimbic Cortex:

Two-way ANOVA revealed a significant effect of treatment ($F(1,34)=4.370$, $p<0.05$) but not fear-conditioning ($F(2,34)=1.403$, $p=0.244$) or fear-conditioning*treatment ($F(2,34)=3.911$, $p=0.056$) on the levels of AEA in the IL. Further post-hoc analysis revealed a significant increase in the levels of AEA following MJN110 treatment in FC rats (FC MJN110 vs. FC VEH, $p<0.05$) (Figure 3.9).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,34)=0.729$, $p=0.399$), treatment ($F(2,34)=2.639$, $p=0.113$), or fear-conditioning*treatment ($F(2,34)=0.170$, $p=0.683$) on the levels of 2-AG in the IL (Figure 3.9).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,34)=0.569$, $p=0.456$), treatment ($F(2,34)=0.304$, $p=0.585$), or fear-conditioning*treatment ($F(2,34)=1.306$, $p=0.261$) on the levels of PEA in the IL (Figure 3.9).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,34)=0.228$, $p=0.636$), treatment ($F(2,34)=0.533$, $p=0.470$), or fear-conditioning*treatment ($F(2,34)=2.089$, $p=0.158$) on the levels of OEA in the IL (Figure 3.9).

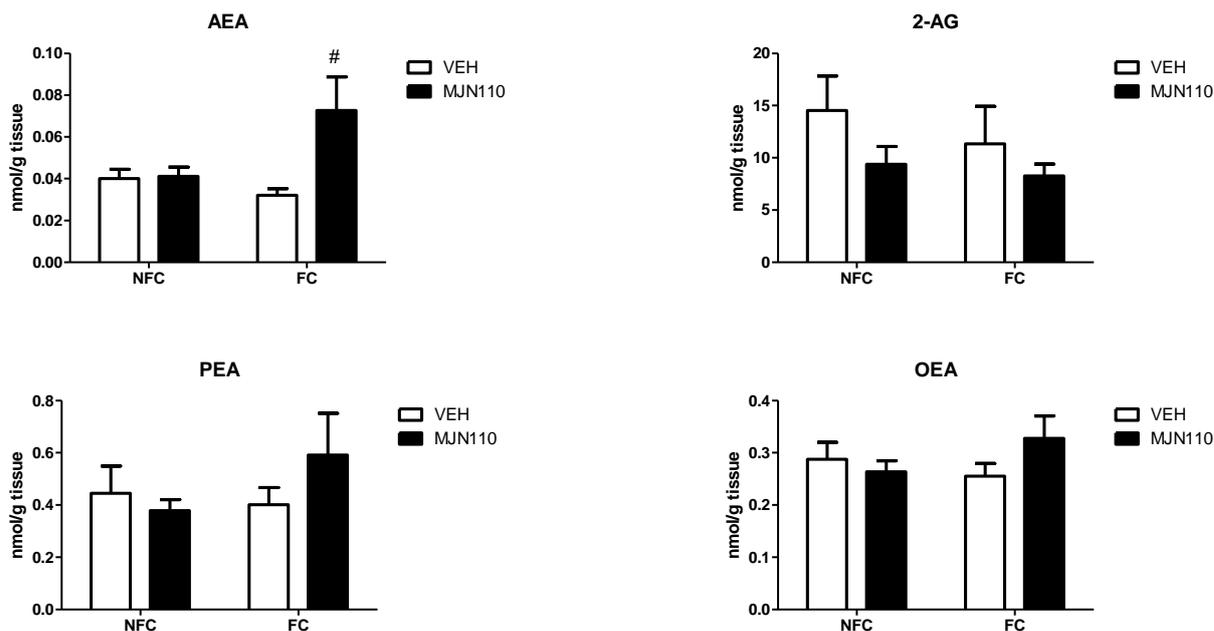


Figure 3.9: Effect of fear-conditioning and MJN110 on AEA, 2-AG, PEA or OEA levels in the IL. # $p < 0.05$ vs FC VEH (Tukey's). All data are expressed as mean \pm SEM (n=9-11 per group); NFC-Non-fear-conditioned; FC-fear-conditioned; VEH-vehicle

Prelimbic Cortex:

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,31)=0.870$, $p=0.358$), treatment ($F(2,31)=0.417$, $p=0.523$), or fear-conditioning*treatment ($F(2,31)=0.034$, $p=0.854$) on the levels of AEA in the PrL (Figure 3.10).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,31)=0.319$, $p=0.576$), treatment ($F(2,31)=2.129$, $p=0.155$), or fear-conditioning*treatment ($F(2,31)=0.932$, $p=0.342$) on the levels of 2-AG in the PrL (Figure 3.10).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,31)=0.422$, $p=0.521$), treatment ($F(2,31)=1.149$, $p=0.292$), or fear-conditioning*treatment ($F(2,31)=0.909$, $p=0.348$) on the levels of PEA in the PrL (Figure 3.10).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,31)=0.372$, $p=0.546$), treatment ($F(2,31)=1.508$, $p=0.229$), or fear-conditioning*treatment ($F(2,31)=0.929$, $p=0.342$) on the levels of OEA in the PrL (Figure 3.10).

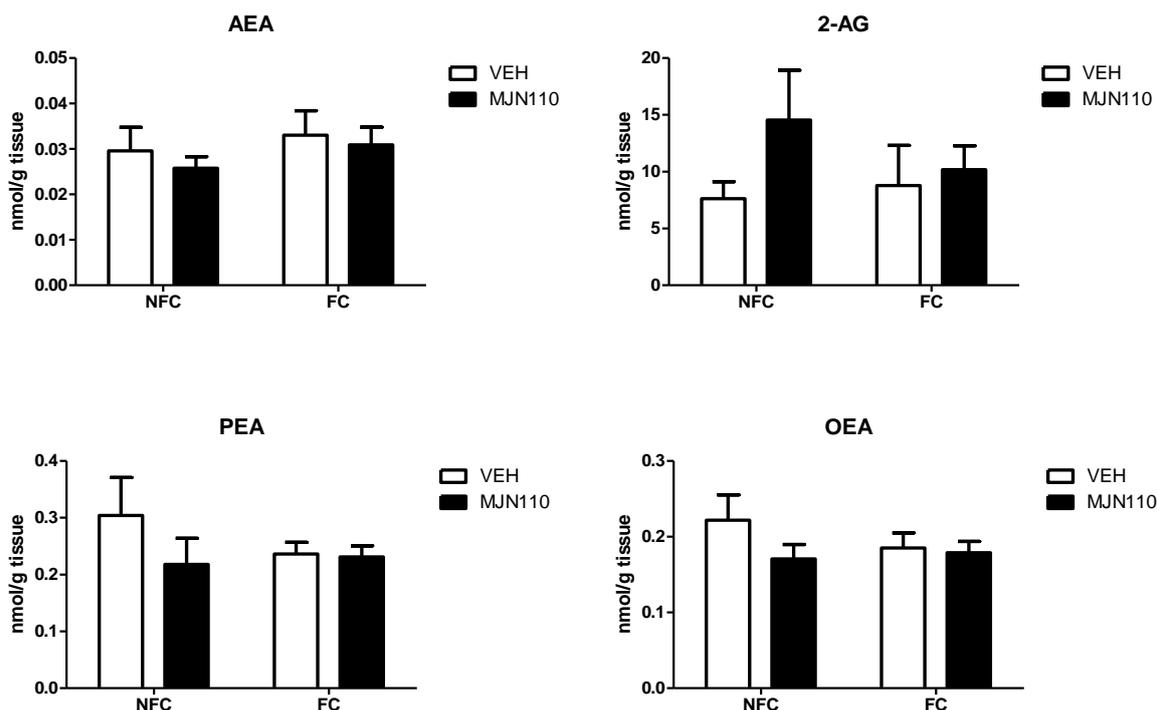


Figure 3.10: Effects of fear-conditioning and MJN110 on AEA, 2-AG, PEA or OEA levels in the PrL. All data are expressed as mean \pm SEM (n=7-11 per group); NFC-Non-fear-conditioned; FC-fear-conditioned; VEH-vehicle

Anterior Cingulate Cortex:

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,33)=0.011$, $p=0.916$), treatment ($F(2,33)=0.051$, $p=0.822$), or fear-conditioning*treatment ($F(2,33)=0.272$, $p=0.605$) on the levels of AEA in the ACC (Figure 3.11).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,33)=0.774$, $p=0.385$), treatment ($F(2,33)=0.064$, $p=0.802$), or fear-conditioning*treatment ($F(2,33)=0.084$, $p=0.774$) on the levels of 2-AG in the ACC (Figure 3.11).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,33)=0.251$, $p=0.620$), treatment ($F(2,33)=0.002$, $p=0.961$), or fear-conditioning*treatment ($F(2,33)=0.993$, $p=0.326$) on the levels of PEA in the ACC (Figure 3.11).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,33)=0.114$, $p=0.738$), treatment ($F(2,33)=0.027$, $p=0.870$), or fear-conditioning*treatment ($F(2,33)=0.931$, $p=0.342$) on the levels of OEA in the ACC (Figure 3.11).

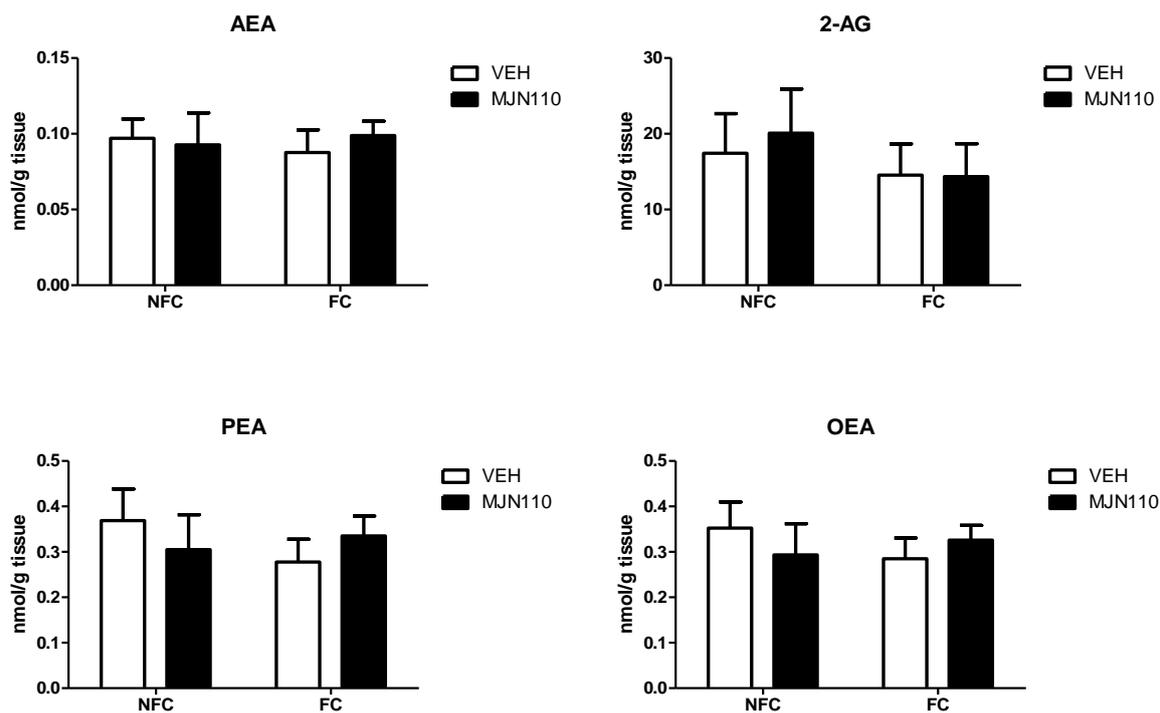


Figure 3.11: Effects of fear-conditioning and MJN110 on AEA, 2-AG, PEA or OEA levels in the ACC. All data are expressed as mean \pm SEM (n=9-10 per group); NFC-Non-fear-conditioned; FC-fear-conditioned; VEH-vehicle

3.4 Discussion

The data presented herein indicate for the first time that a MAGL substrate in the IL regulates fear-pain interactions. In the absence of contextual fear-conditioning, administration of the MAGL inhibitor MJN110 alone or in combination with the CB₁ receptor antagonist AM251 had no effect on formalin-evoked nociceptive behaviour. Re-exposure of vehicle-treated rats to a context previously paired with footshock significantly reduced formalin-evoked nociceptive behaviour (i.e. induced FCA) and increased contextually induced freezing and 22kHz ultrasonic vocalisation in the presence of nociceptive tone. Microinjection of the MAGL inhibitor MJN110 into the IL attenuated the expression of FCA, increasing formalin-evoked nociceptive behaviour in FC rats, an effect unopposed upon co-administration with AM251. Treatment with MJN110 alone or in combination with AM251 decreased the distance moved in FC rats compared with FC VEH rats. Fear-conditioning also significantly increased the duration of rearing in rats, an effect blocked by MJN110 alone or in combination with AM251. Mass spectrometry revealed a significant increase in the levels of AEA in the IL of FC MJN110-treated rats compared to FC VEH-treated rats. Neither FC nor intra-IL administration of MJN110 were associated with alterations of endocannabinoids or related n-acyl ethanolamines in the PrL or ACC at the end of the 30-minute testing. These data suggest that MAGL in the IL is an important neural substrate in regulating the expression of FCA.

In the present study, VEH-treated FC rats exhibited a reduction in formalin-evoked nociceptive behaviour upon re-exposure to a context previously paired with an aversive stimulus (i.e. footshock), confirming the expression of FCA. This finding is consistent with chapter 2 along with a number of previous studies, within our own group and others, investigating FCA (Fanselow, 1984, Finn et al., 2004, Olango et al., 2012, Rea et al., 2013, Roche et al., 2007, Helmstetter, 1992, Helmstetter and Bellgowan, 1993, Rea et al., 2014a, Rea et al., 2009).

MAGL inhibition via MJN110 attenuated FCA and increased the expression of formalin-evoked nociceptive behaviour in FC rats in the last 10-minutes of the testing period while having no effect on contextually induced freezing and 22kHz ultrasonic vocalisation in the presence of nociceptive tone. This effect was not blocked following co-administration with the CB₁ receptor antagonist AM251. These findings suggest that FCA is suppressed by a MAGL substrate, possibly 2-AG, and provide further evidence that the IL is an important neural substrate for endocannabinoid-related FCA (Rea et al., 2018), in addition to the ventral hippocampus (Ford et al., 2011), dlPAG (Olango et al., 2012) and the BLA (Rea et al., 2013, Roche et al., 2007) and PrL (Rea et al., 2018). Unlike the present study, intra-dlPAG MAGL

inhibition via URB602 enhanced SIA in a CB₁-dependent manner (Hohmann et al., 2005), indicating a region-specific effect of MAGL substrates on SIA and FCA.

The effects of MJN110 alone were not blocked by co-administration of AM251. These results suggest that the MAGL substrate modulates expression of FCA via a non-CB₁ receptor target. Moreover, the combination of MJN110+AM251 attenuated FCA for the entire 30-minute testing period, as indicated by the 30-minute time bin data (Figure 3.3) and the temporal analysis of the data in 10-minute time bins (Figure 3.4), while the effect of MJN110 alone was only evident during the last 10-minutes of testing. A previous study from our group investigated the effects of direct microinjection of AM251 into the IL on the expression of FCA and found that CB₁ receptor antagonism attenuated the expression of FCA in the first 10-minutes of testing only (Rea et al., 2018). This effect of AM251 may account for the robust and more prolonged attenuation of FCA seen in this study with MJN110 and AM251 administered in combination. Although 2-AG is a ligand at CB₁ receptors, by antagonising this receptor with AM251 and blocking MAGL activity, 2-AG may then act on non-CB₁ receptor targets, resulting in a robust attenuation of FCA. Such non-CB₁ targets (Baggelaar et al., 2018) could include CB₂ (Gonsiorek et al., 2000, Sugiura et al., 2000), GABA_A (Sigel et al., 2011), PPARs (Bouaboula et al., 2005), Adenosine A₃ (Lane et al., 2010), TRPV1 (Yusaku et al., 2008) and/or GPR55 (Ryberg et al., 2007).

Our group has previously investigated the role of FAAH in the IL in FCA. Intra-IL FAAH inhibition via URB597 and CB₁ receptor blockade via AM251, alone or in combination, attenuated the expression of FCA (Rea et al., 2018). Moreover, Rea et al., suggest that the effects of URB597 and AM251 in the IL are not compatible with the idea that endocannabinoid-CB₁ signalling mediates FCA, (FAAH inhibition vs. CB₁ receptor blockade), and suggest that a non-CB₁ receptor target may be mediating the effects of URB597 in the IL (Rea et al., 2018). This is comparable to the results herein that suggest a MAGL substrate is acting via a non-CB₁ receptor target to attenuate FCA. These results also indicate a common role for FAAH substrates and 2-AG in the IL as both attenuated FCA. Previous studies have demonstrated that 2-AG administration into the ventral hippocampus attenuates formalin-evoked nociceptive behaviour in the absence but not presence of contextual fear (Rea et al., 2014a). In the present study, MAGL inhibition had no effect on formalin-evoked nociceptive behaviour in NFC rats but increased nociceptive responding in FC rats in the final 10-minutes of the testing period. These results suggest region-specific effects of 2-AG supraspinally on the expression of formalin-evoked nociceptive behaviour.

Fear-conditioning significantly increased the duration of the fear-related behaviour in the form of increased duration of freezing and 22kHz ultrasonic vocalisation in VEH-treated rats. Intra-IL administration of MJN110 alone or in combination with AM251 had no effect on the expression of fear-related behaviours in FC rats. In contrast, Rea et al., found that either URB597 or AM251 alone or in combination attenuated contextually induced freezing behaviour when injected into the IL, indicating that the endocannabinoid-mediated effects on FCA and contextually induced fear behaviour were not dissociable. In the present study intra-IL MJN110 and MJN110+AM251 had no effect on contextually induced freezing behaviour while attenuating FCA, indicating that the effects on freezing and FCA were dissociable. These results corroborates our previous studies (Finn et al., 2004, Rea et al., 2013, Roche et al., 2010, Roche et al., 2007) and those of others (Helmstetter and Fanselow, 1987, Kinscheck et al., 1984), demonstrating that FCA can be altered independently of the expression of fear-related behaviour in the presence of nociceptive tone. While FAAH and MAGL substrates both attenuate the expression of FCA, they seem to have differing effects on the expression of fear-related behaviours in the IL.

Intra-IL administration of MJN110 decreased the distance moved in FC rats compared to VEH. This effect was not blocked by co-administration with AM251, indicating a role for a non-CB₁ receptor. The drug had no effect on the distance moved exhibited by NFC rats so the decrease in distance moved in FC rats was likely not due to an impairment in locomotor activity. Decreased distance moved in these rats may relate to the increase in formalin-evoked nociceptive behaviour seen upon treatment with either MJN110 or MJN110+AM251. It is possible the rats spend more time immobile with their formalin-injected paw elevated or spend more time in the one spot licking or biting the injected paw. Similarly, fear-conditioning increased the duration of rearing in VEH-treated rats, an effect blocked by intra-IL MJN110 or MJN110+AM251. Rearing has been used as a measure of active coping in response to fearful and stressful stimuli (Gozzi et al., 2010, Metna-Laurent et al., 2012). It is possible that upon fear-conditioning rats exhibit both active and passive coping strategies in the form of freezing and rearing. Treatment with MJN110 or MJN110+AM251 may attenuate active coping (rearing) but not passive coping (freezing) by acting at a receptor other than CB₁. Conditional mutant mice lacking CB₁ receptors from cortical glutamatergic neurons favoured passive coping responses upon fear-conditioning while CB₁ receptor deletion from GABAergic brain neurons led to the opposite phenotype, characterized by the predominance of active coping (Metna-Laurent et al., 2012). It is possible that in the present chapter MAGL substrates act on

a non-CB₁ receptor on GABAergic neurons to decrease active coping without affecting passive coping in fear-conditioned rats. Rearing is a type of exploratory behaviour and has been used as a measure of anxiety in multiple paradigms including the light dark box test (Costall et al., 1989, Crawley et al., 1984), the open field test (Carli et al., 1989, Lamprea et al., 2008) and the elevated plus maze test (Lepicard et al., 2000, Escorihuela et al., 1999). However, whether rearing behaviour is a robust measure of anxiety-related behaviour remains to be determined (Ennaceur, 2014, Seibenhener and Wooten, 2015), with some studies claiming that increased rearing correlates with increased anxiety levels (Panickar and McNaughton, 1991, Borta and Schwarting, 2005, Schwarting et al., 1998) and others suggesting decreased rearing indicates increased anxiety levels (Costall et al., 1987, Costall et al., 1989, Escorihuela et al., 1999). Our study implies that increased rearing may be indicative of increased anxiety as FC VEH-treated rats, expressing increased conditioned freezing behaviour and FCA, also express increased rearing. As it is also an exploratory behaviour increased rearing behaviour could imply that the rats are searching for an escape route out of the arena they previously received footshock in. MJN110 attenuates both FCA and the increased duration of rearing, an effect not blocked by co-administration with AM251, suggesting a potential anxiolytic role for a MAGL substrate in the IL via a non-CB₁ related receptor.

Overall, the above results suggest that a MAGL substrates in the IL dose not play a role in fear-related behaviours (freezing and 22kHz ultrasonic vocalisation) in the presence of nociceptive tone but may elicit anxiolytic-like effects. 2-AG and MAGL inhibition has previously been shown to have anxiolytic-like effects in the elevated zero and plus maze tests, marble burying and restraint stress tests in mice and rats respectively (Busquets-Garcia et al., 2011, Kinsey et al., 2011b, Sciolino et al., 2011, Sumislawski et al., 2011). Systemic 2-AG augmentation is associated with stress resilience while 2-AG depletion is associated with increased stress susceptibility in mice (Bluett et al., 2017). However, certain studies have also indicated anxiogenic-like effects of 2-AG. Llorente-Berzal et al., suggest that while AEA is anxiolytic and mediates fear relief, 2-AG promotes the expression of conditioned fear via CB₁ in auditory-cued fear conditioning in mice (Llorente-Berzal et al., 2015). Similarly, Hartley et al., found that 2-AG signalling impairs the expression of short-term fear-extinction in the auditory cue fear-conditioning paradigm in mice (Hartley et al., 2016). It is worth noting that the anxiolytic/anxiogenic effects in these published studies were observed in the absence of a nociceptive stimulus unlike the present study where MAGL inhibition had no effect on contextual fear in the presence of nociceptive tone but altered rearing behaviour in this

environment. Further studies are required to determine the anxiolytic/anxiogenic effects of 2-AG in the IL in the absence of nociceptive tone.

In an attempt to determine the mechanism underlying the effects of intra-IL administration of MJN110 on the expression of FCA and fear-related behaviours we measured levels of the endocannabinoids AEA and 2-AG and related N-acylethanolamines OEA and PEA locally in the IL and surrounding PrL and ACC tissue. Fear-conditioning and intra-IL MJN110 did not significantly alter tissue levels of endocannabinoids or related N-acylethanolamines in the PrL or ACC. Fear-conditioning and intra-IL MJN110 also had no effect on the levels of 2-AG, PEA and OEA in the IL but intra-IL MJN110 did increase AEA levels in the IL of FC rats compared to FC VEH treated rats. MJN110 is a MAGL inhibitor so we would expect to see an increase in 2-AG levels in the IL. In contrast to our own results, Sticht et al., found that same dose of MJN110 (2 μ g/ μ l) injected directly into the visceral insular cortex increased the levels of 2-AG but not AEA in a rat model of nausea (Sticht et al., 2016). MJN110 is suggested to potently inhibit 2-AG hydrolysis but have no effect on AEA hydrolysis (Niphakis et al., 2013). The brains were removed and frozen 45-minute post microinjection in our study and thus it is possible that 2-AG levels were increased in the IL at an earlier timepoint. While MJN110 increased the levels of AEA there appears to be no evidence from the literature suggesting that MAGL substrates modulate AEA levels. FAAH inhibition in the IL (with a potential subsequent increase in endogenous AEA tone) also attenuates the expression of FCA (Rea et al., 2018) and suggests a complementary role for FAAH and MAGL substrates in the IL. It is possible that increased 2-AG signalling via MAGL inhibition can modulate the levels of AEA in the IL to aid in the attenuation of FCA. DAGL KO mice exhibit decreased levels of both 2-AG and AEA in the hippocampus, amygdala and cortex, while in the striatum only the levels of 2-AG were decreased. Moreover, MAGL inhibition via JZL184 increased the levels of 2-AG and normalised the levels of AEA in KO mice (Jenniches et al., 2016, Gao et al., 2010). The authors of this study suggest that the levels of 2-AG may influence the levels of AEA in a region-specific manner although the mechanism for this is not known. It is possible that the levels of 2-AG may influence AEA synthesis or degradation through an unknown mechanism. 2-AG and AEA are substrates or products for the same or related enzymes which may contribute to the impact on AEA levels following MAGL inhibition. Future studies looking at dual FAAH and MAGL inhibitors in the IL or FAAH/MAGL knockout studies could shed light on the role of the two enzymes and their substrates in the expression of FCA and endogenous fear in the IL of rats. It is also possible that the effects of MJN110 are mediated by elevation

of the MAGL substrate 2-oleoylglycerol. This is a biologically active MAGL substrate derived from DAGL precursors (Dinh et al., 2002). Although little is known about its in-vivo activity, it was found to act as an endogenous ligand at GPR199 in the small intestine (Hansen et al., 2011).

In conclusion, the present study provides new evidence to support a role for a MAGL substrate in the IL in the expression of FCA via a non-CB₁ receptor. When considered alongside previous work, the data suggest that FAAH and MAGL substrates in the IL may have similar effects on the expression of FCA but differing effects on the expression of fear-related behaviours. Intra-MJN110 increased the levels of AEA but not 2-AG in the IL, thus, the precise mechanisms underlying the behavioural effects observed in this study require further investigation. Therefore, further studies are required to elucidate the receptors and molecular mechanisms by which MAGL inhibition exerts effects in the IL.

Chapter 4 : The role of 2-AG in the prelimbic cortex in formalin-evoked nociceptive behaviour, fear-conditioned analgesia and conditioned fear in the presence of nociceptive tone in rats

4.1 Introduction

The PrL of rodents is believed to be homologous to the dorsolateral PFC of primates and Brodmann area 32 in humans. The PrL is slightly larger than the IL (described in chapter 3) in rodents, located dorsal to the IL and ventral to the ACC, extending from the frontal pole through the genu of the corpus callosum underneath the ACC (Ongur and Price, 2000). Like the IL, the PrL has numerous projections, including but not limited to other subregions of the mPFC (IL and ACC), mediodorsal nucleus of the thalamus, olfactory forebrain, ventral tegmental area, anterior piriform cortex, dorsal raphe nucleus, nucleus accumbens shell and core, amygdala, PAG and hippocampus (Vertes, 2004). The projections to and from the PrL reflect its numerous functions including, limbic cognitive functions, decision-making, goal-directed behaviour and working memory, pain modulation, anxiety responses to stress, and fear expression (Vertes, 2004).

The PrL has an important role in fear-related behaviour, in particular the regulation of fear expression. Early lesion studies indicated that dorsal PrL lesions produced increases in fear expression, suggesting a role for the dorsal PrL in fear suppression (Holson, 1986, Morgan and LeDoux, 1995). Electrophysiological studies have since indicated sustained neuronal activity in the PrL was associated with ongoing freezing behaviour following auditory fear-conditioning that dissipated with extinction (Burgos-Robles et al., 2009). In rats with extinction deficits following exposure to chronic restraint, PrL neurons show prolonged activity compared to control rats (Wilber et al., 2011). Increased PrL activity has also been seen in mice with impaired extinction retrieval (Fitzgerald et al., 2014). Pharmacological inactivation of the PrL via tetrodotoxin during fear learning or expression reduces freezing in both auditory and contextual fear conditioning in rats, an effect not seen when the PrL is inactivated before conditioning (Corcoran and Quirk, 2007). Similarly, inactivation of the rat PrL with muscimol (GABA_A receptor agonist) prior to extinction training impairs the expression of freezing (Laurent and Westbrook, 2009, Sierra-Mercado et al., 2011). This implies a key role for the PrL in the expression but not acquisition of fear. The PrL may also play a role in aversive

learning. Microstimulation of the PrL increased the expression of conditioned fear and prevented extinction following auditory fear conditioning paired with electrical stimulation (Vidal-Gonzalez et al., 2006). The above information paired with the fact that, like the IL, the PrL projects to other areas involved in fear-related behaviour (amygdala and hippocampus) (Vertes, 2004), suggests a role for the PrL in the top-down regulation of fear-related processes.

The role of the PrL in pain is not as well characterised. Lesion of the PrL attenuated CFA-induced chronic inflammatory pain and anxiety related behaviour in rats when lesioned contralateral but not ipsilateral to the injected CFA paw (Wang et al., 2015). Contralateral activation of PrL excitatory neurons, via optogenetics, is antinociceptive in mice subject to a similar chronic inflammatory pain paradigm with complete Freund's adjuvant (Wang et al., 2015). Electrophysiological recordings (in brain slices) suggest that increased PrL pyramidal neuron excitability may contribute peripheral inflammatory pain in the CFA model in mice (Wu et al., 2016). Intra-PrL administration of ethanol has been shown to lower mechanical pain threshold in the Von Frey test in rats without altering thermal pain sensitivity, an effect mediated by GABA_A receptors (Geng et al., 2016). mGluR5 in the PrL may also contribute to expression of neuropathic pain. PET analysis with an mGluR5 selective radiotracer revealed an upregulation of mGluR5 in the PrL in a rat model of neuropathic pain. Pharmacological blockade of mGluR5 in the PrL via MPEP alleviated neuropathic mechanical allodynia in spinal nerve ligated rats (Chung et al., 2017). These studies suggest a role for the PrL in pain-related processes, in particular inflammatory and neuropathic pain, and highlight a role for both GABA and glutamate receptors. Inactivation of the PrL with muscimol prevented the acquisition and expression of formalin-induced conditioned place avoidance in rats (Jiang et al., 2014). Whole cell patch clamp techniques suggest that spared nerve injury reduces the activity of prelimbic pyramidal cells via a GABAergic feed-forward inhibition mechanism in mice (Zhang et al., 2015). Optogenetic manipulation of mice revealed that activation of parvalbumin-positive interneurons in the PrL enhances pain behaviour and the aversive state, whereas, inhibition alleviates pain and the aversive state in the conditioned-place preference test (Zhang et al., 2015). These studies suggest a role for the PrL in the affective component of pain.

The endocannabinoid system plays a key role in pain and fear and their interactions (Corcoran et al., 2015, Lutz et al., 2015). The endocannabinoid system in the PrL has been shown to play a role in unconditioned SIA and in FCA. SIA, induced via GABA_A receptor antagonism in the dorso/ventro medial hypothalamus was attenuated following intra-PrL administration of the

CB₁ receptor antagonist AM251 at multiple doses in rats (Freitas et al., 2013). In agreement, our laboratory has shown that the FAAH inhibitor URB597 prolongs FCA when injected into the PrL while AM251 attenuates it (Rea et al., 2018).

As discussed in chapter 3, 2-AG plays a role in the regulation of fear, anxiety and pain (Bluett et al., 2017, Busquets-Garcia et al., 2011, Sciolino et al., 2011, Sumislowski et al., 2011, Rea et al., 2014a, Wilkerson et al., 2017, Llorente-Berzal et al., 2015, Hartley et al., 2016). However, no studies to date have investigated the role of 2-AG and MAGL in the PrL in formalin-evoked nociceptive behaviour, FCA and conditioned fear in the presence of nociceptive tone,

This chapter will assess the role of 2-AG in the PrL in formalin-evoked nociceptive behaviour, FCA and conditioned fear in the presence of nociceptive tone with the following hypothesis: MAGL inhibition in the PrL will enhance the expression of FCA while having no effect on formalin-evoked nociceptive behaviour or conditioned fear in the presence of nociceptive tone.

. Therefore, the overall aims of the study described in this chapter were:

- To determine the role of 2-AG in the PrL in expression of formalin-evoked nociceptive behaviour, FCA and fear behaviour in the presence of formalin-evoked nociceptive tone. This was achieved by examining the effects of intra-PrL administration of the MAGL inhibitor, MJN110 alone or in combination with the CB₁ receptor antagonist/inverse agonist, AM251, on these phenomena in rats.
- To examine if behavioural changes are associated with alterations in endocannabinoid levels in the PrL and neighbouring mPFC regions using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

4.2 Materials and Methods

4.2.1 Animals:

Male Lister-Hooded rats (260–320g on day of behavioural testing); Charles River, Margate, Kent, UK) were used. Before surgery, animals were housed 2-3 per flat bottomed cage (L: 45 x H: 20 x W: 20cm) containing wood shavings as bedding (LBS, Surrey, UK). Animals were kept at a constant temperature ($21^{\circ}\text{C} \pm 2^{\circ}\text{C}$) under standard lighting conditions (12:12h light–dark, lights on from 0700 to 1900h). Experiments were carried out during the light phase between 0800 and 1700h. Food (14% Harlan-Teklad-2014 Maintenance Diet, Harlan Laboratories, Belton, Loughborough, UK) and water were provided *ad libitum*. The experimental protocol was carried out after approval by the Animal Care and Research Ethics Committee, National University of Ireland Galway, under license from the Health Products Regulatory Authority and in compliance with the European Communities Council directives 86/609 and 2010/63 and conformed to the ARRIVE guidelines.

4.2.2 Cannulae implantation:

See Chapter 2 section 2.2.2 for details on cannula implantation via stereotaxic surgery.

Briefly, stainless steel guide cannulae (Plastics One Inc., Roanoke, Virginia, USA) were stereotaxically implanted bilaterally 1mm above the right and left PrL (AP + 2.4mm relative to bregma, ML \pm 1.5mm relative to bregma and at a 12° angle and DV – 2.3mm from dura, toothbar set at -3.0mm).

Following surgery animals were singly housed and a single daily dose of enrofloxacin (2.5mg/kg, s.c., Baytril; Bayer Ltd., Dublin, Ireland) was administered for the following four days. A minimum of 6 days was allotted to allow rats to recover before any experimentation commenced. During this period, the rats were handled, stylets checked, and their body weight and general health monitored.

4.2.3 Chemicals and drug preparation:

See chapter 3 section 3.2.3.

4.2.4 Experimental procedure:

The experimental procedure was identical to that described in chapter 2 section 2.2.4.

On test days, rats received intra-PrL microinjection (0.3µl per side) of either vehicle (VEH; 100% DMSO), the MAGL inhibitor MJN110 (2µg/µl) or the CB₁ receptor antagonist AM251+MJN110 (AM251:2mM, MJN110: 2µg/µl). A group receiving AM251 alone was not included because the effects of intra-PrL administration of AM251 alone on the same behavioural parameters in an identical experimental design were investigated by Rea et al., 2018.

After 30-minutes in the arena, rats were removed and euthanised by decapitation. Brains and spinal cords were rapidly removed within a 2-minute timeframe, snap-frozen on dry ice and stored at -80°C. After decapitation, 0.3µl of 2% fast green dye (dissolved in DMSO) was also injected into the left and right PrL for post-mortem confirmation of microinjection sites during cryo-sectioning.

This design resulted in 6 experimental groups (Starting n=12 per group for surgery; final n=8-11 per group for data analysis) as illustrated in Table 4.1.

Group	Conditioning	Formalin i.pl.	Drug/Vehicle	N
1	FC	Formalin	100% DMSO	8
2	NFC	Formalin	100% DMSO	9
3	FC	Formalin	MJN110	8
4	NFC	Formalin	MJN110	10
5	FC	Formalin	MJN110+AM251	10
6	NFC	Formalin	MJN110+AM251	11

Table 4.1: Summary of experimental groups: i.pl., intraplantar; FC, fear-conditioned; NFC, non – fear-conditioned

4.2.5 Brain removal:

See chapter 2 section 2.2.5.

4.2.6 Behavioural analysis:

See chapter 2 section 2.2.6.

4.2.7 Histological verification of intracerebral injection sites:

See chapter 2 section 2.2.7.

4.2.8 Tissue isolation by Palkovits punch:

See chapter 3 section 3.3.8.

4.2.9 Measurement of levels of endocannabinoids in the medial prefrontal cortex:

See chapter 2 section 2.2.9 with the same amendments as chapter 3 section 3.3.9.

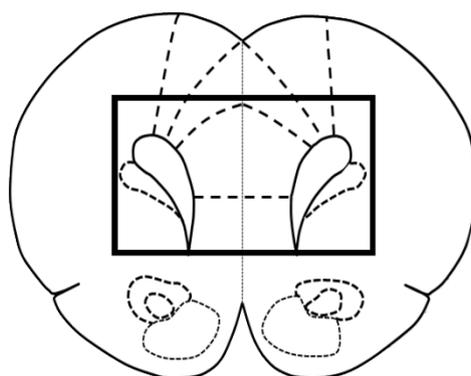
4.2.10 Statistical analysis:

The IBM SPSS statistical software package (SPSS v23.0 for Microsoft Windows; Chicago, IL, USA) was used to analyse all data. Normality and homogeneity of variance were assessed using Shapiro-Wilk's and Levene's test, respectively. All data except the duration of freezing, 22kHz ultrasonic vocalisation and defecation were found to be parametric. Behavioural data and mass spectrometry data were analysed by 2-way ANOVA, with fear-conditioning and drug treatment as factors. Time course behavioural data were analysed by 2-way repeated measures ANOVA with time as the within-subjects factor and fear-conditioning and drug treatment as the between-subjects factors. Sphericity was tested using Mauchly's Test for Sphericity. If sphericity was violated a Greenhouse-Geisser correction was used. Post-hoc pairwise comparisons were made with Tukey's test when appropriate. Non-parametric data was analysed using Kruskal Wallis analysis of variance and post-hoc analysis performed using Dunns-Bonferroni pairwise comparisons when appropriate. For repeated measures non-parametric data, Kruskal Wallis was performed followed by Dunn-Bonferroni post-hoc where appropriate. Data were considered significant when $p < 0.05$. Results are expressed as group means \pm SEM or median with interquartile range.

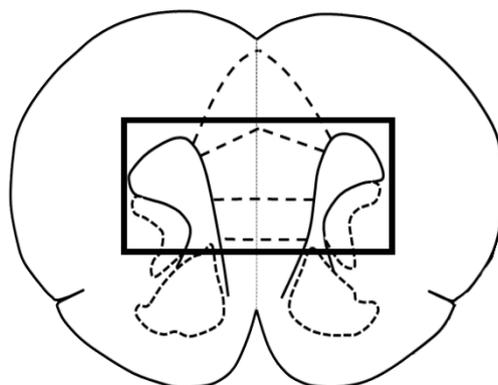
4.3 Results

4.3.1 Histological verification of microinjection sites

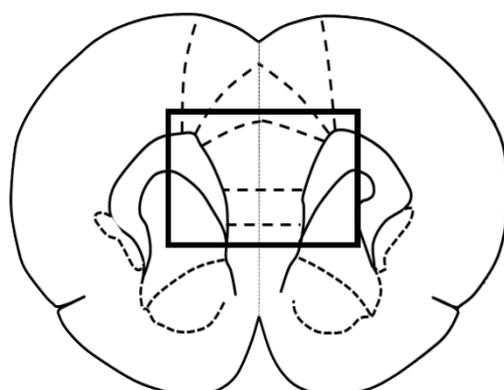
85% of the injections were within the borders of the PrL (Figure 4.1) with the remaining 15% placed just before the PrL in the medial orbital cortex or lateral to the PrL in the corpus callosum. The data analysed were derived only from rats in which bilateral injections were correctly positioned in the PrL.



+3.2 mm from Bregma



+2.7 mm from Bregma



+2.2 mm from Bregma

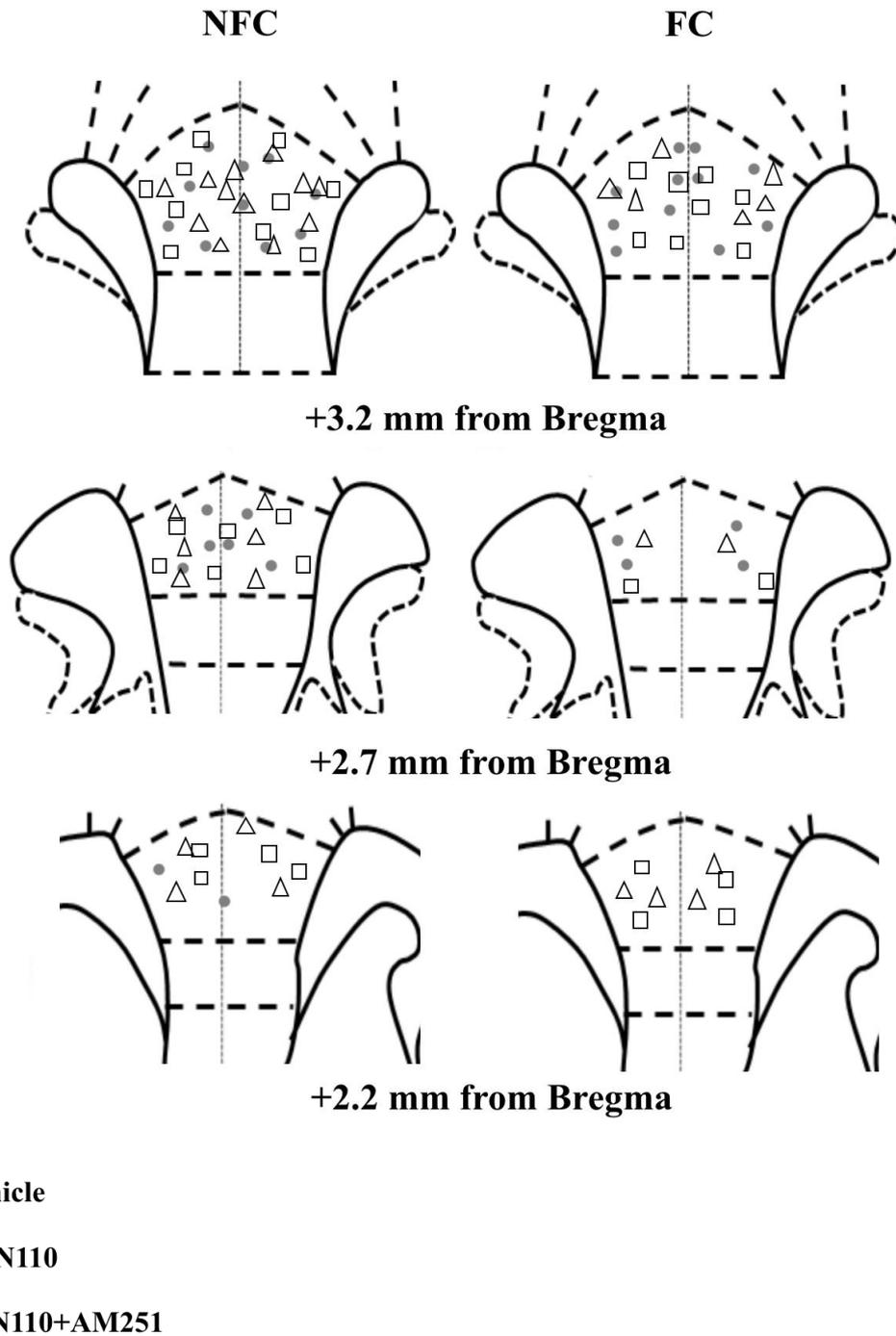


Figure 4.1: Histological verification of injector site location. Adapted from Paxinos & Watson (1998). NFC: Non-fear-conditioned; FC: Fear-conditioned

4.3.2 Effects of intra-PrL administration of MJN110 or MJN110+AM251 on formalin-evoked nociceptive behaviour and FCA

Intra-plantar injection of formalin induced a nociceptive response in the form of elevation, licking, biting and shaking of the injected right hind-paw. Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,50)=1.580$, $p=0.215$), treatment ($F(2,50)=0.472$, $p=0.627$) or fear-conditioning*treatment ($F(2,50)=0.389$, $p=0.680$) on the change in hind paw diameter pre- versus post-formalin injection (Figure 4.2).

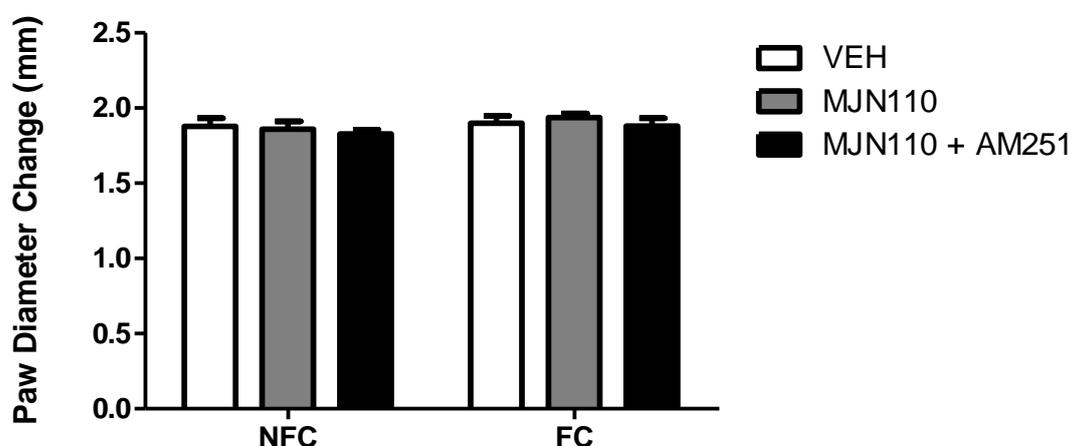


Figure 4.2: Effects of fear-conditioning and bilateral administration of MJN110 or MJN110+AM251 directly into the PrL on paw diameter change (mm) pre and post formalin injection (oedema). All data are expressed as mean \pm SEM ($n=8-11$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Two-way ANOVA revealed a significant effect of fear-conditioning ($F(1,50)=52.014$, $p<0.001$), treatment ($F(2,50)=9.877$, $p<0.001$) and fear-conditioning*treatment ($F(2,50)=17.176$, $p<0.001$) on CPS over the course of the 30-minute testing period. Further post-hoc analysis revealed that re-exposure of rats to the arena previously paired with footshock resulted in a significant reduction in formalin-evoked nociceptive behaviour, confirming the expression of FCA (FC VEH vs. NFC VEH, $p<0.001$). Intra-PrL administration of MJN110 or MJN110+AM251 significantly attenuated the expression of FCA over the course of the 30-minute trial (FC VEH vs. FC MJN110 or FC MJN110+AM251, $p<0.001$). There were no effects of any of the drug treatments in NFC rats (Figure 4.3).

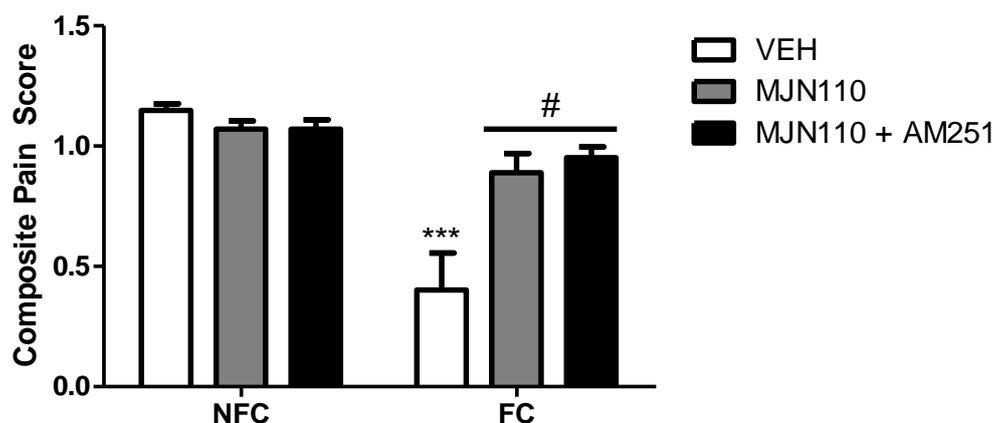


Figure 4.3: Effects of fear-conditioning and bilateral administration of MJN110 and MJN110+AM251 directly into the PrL on formalin-evoked nociceptive behaviour in rats over the full 30-minute testing period. *** $p < 0.001$ FC vs. NFC; # $p < 0.001$ vs FC VEH (Tukey's). All data are expressed as mean \pm SEM (n=8-11 per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Temporal analysis of the data subdivided into 10-minute time bins and using 2-way repeated measures ANOVA revealed a significant main effect of fear-conditioning ($F(1,50)=38.872$, $p < 0.001$), treatment ($F(2,50)=6.830$, $p < 0.01$) and fear-conditioning*treatment ($F(2,50)=12.371$, $p < 0.001$) on CPS over the course of the testing period. Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated ($\chi^2(2)=35.746$, $p < 0.001$) and therefore a Greenhouse-Geisser correction was used. There was a significant main effect of time ($F(1.32,65.88)=17.794$, $p < 0.001$) and time*fear-conditioning ($F(2.64,65.88)=10.487$, $p < 0.001$) but not time*treatment ($F(2.64,65.88)=1.157$, $p=0.330$) or time*fear-conditioning*treatment ($F(2.64,65.88)=0.244$, $p=0.841$) on CPS with respect to time. Further post-hoc analysis revealed a significant reduction in formalin-evoked nociceptive behaviour in FC VEH treated rats for the entire 30-minute testing period (FC VEH vs. NFC VEH, 0-10mins, 10-20mins, 20-30mins, $p < 0.001$) confirming the expression of FCA throughout the trial. Intra-PrL administration of MJN110, alone or in combination with AM251, significantly attenuated the expression of FCA, in the final 20 minutes of the trial (FC VEH vs. FC MJN110 or FC MJN110+AM251, 10-20mins, 20-30mins, $p < 0.001$), with similar

trends in the first 10-minutes of the trial. There were no effects of these drug treatments in NFC animals (Figure 4.4).

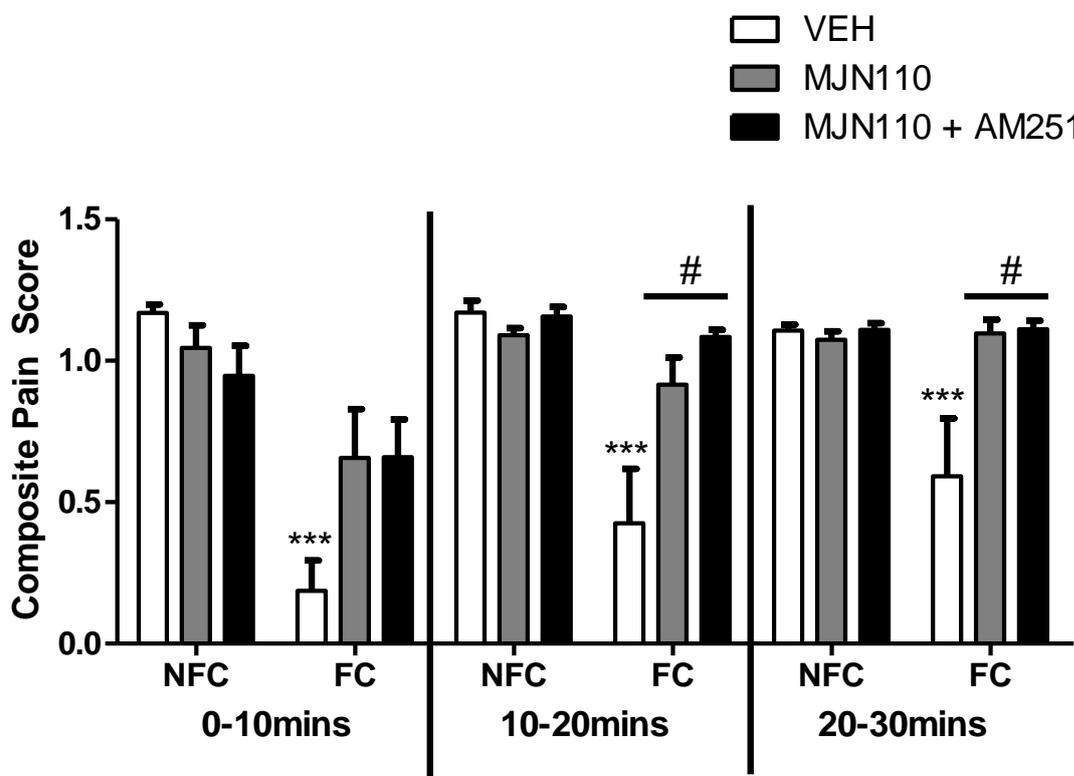


Figure 4.4: Effects of fear-conditioning and bilateral administration of MJN110 and MJN110+AM251 directly into the PrL on formalin-evoked nociceptive behaviour in rats over the full 30-minute testing period subdivided into 10-minute time bins. *** $p < 0.001$ FC vs. NFC; # $p < 0.001$ vs FC VEH (Tukey's). All data are expressed as mean \pm SEM ($n=8-11$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

4.3.3 Effects of intra-PrL administration of MJN110 or MJN110+AM251 on expression of conditioned fear behaviour in the presence of formalin-evoked nociceptive tone

Freezing Behaviour:

Kruskal Wallis comparisons revealed a significant main effect on the duration of freezing ($\chi^2(5)=47.911$, $p < 0.001$) over the course of the 30-minute testing period. Further post-hoc analysis using Dunn-Bonferroni revealed that re-exposure of rats to the arena previously paired with footshock resulted in a significant increase in the duration of freezing (FC VEH vs. NFC

VEH, $p < 0.001$) over the course of the 30-minute testing period. Intra-PrL MJN110 alone or in combination with AM251 had no effect on the duration of freezing (Figure 4.5).

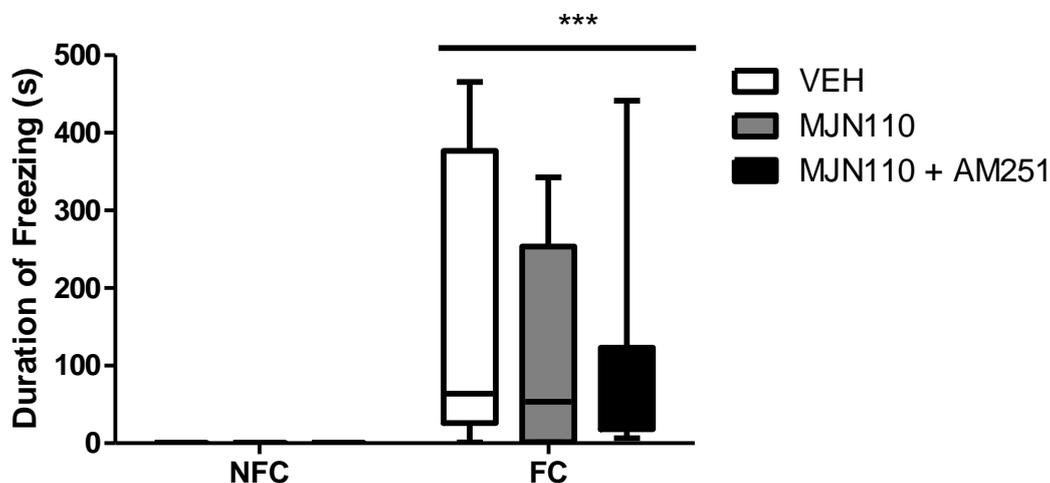


Figure 4.5: Effects of fear-conditioning and bilateral administration of MJN110 and MJN110+AM251 directly into the PrL on the duration of freezing behaviour in rats over the full 30-minute testing period. *** $p < 0.001$ FC vs. NFC; (Dunn-Bonferroni). All data are expressed as median with interquartile range ($n = 8-11$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Analysis of the data subdivided into 10-minute time bins using Kruskal-Wallis revealed a significant between-group effect on the expression of freezing at 0-10mins ($\chi^2(5) = 43.159$, $p < 0.001$) but not 10-20mins ($\chi^2(5) = 10.190$, $p = 0.070$) or 20-30mins ($\chi^2(5) = 8.715$, $p = 0.121$). Post-hoc analysis using the Dunn-Bonferroni test revealed that re-exposure of rats to the arena previously paired with footshock resulted in a significant increase in the duration of freezing in the first 10-minutes of the trial (FC VEH vs. NFC VEH; 0-10mins, $p < 0.01$). Intra-PrL MJN110 alone or in combination with AM251 had no effect on the duration of freezing (Figure 4.6).

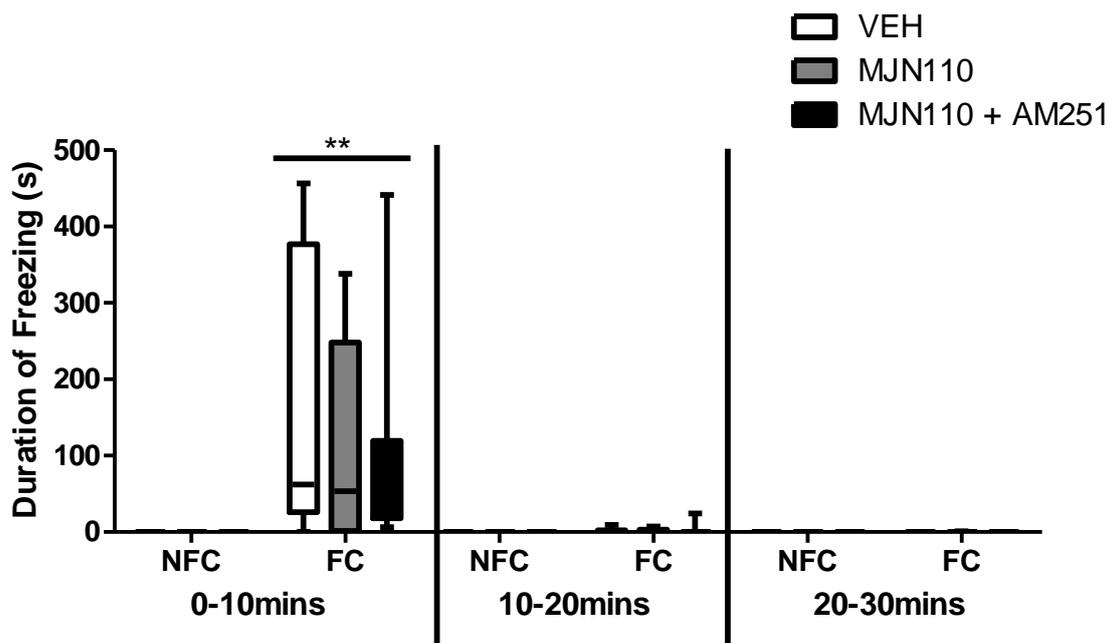


Figure 4.6: Effects of fear-conditioning and bilateral administration of MJN110 and MJN110+AM251 directly into the PrL on the duration of freezing behaviour in rats over the full 30-minute testing period subdivided into 10-minute time bins. ** $p < 0.01$ FC vs. NFC; (Dunn-Bonferroni). All data are expressed as median with interquartile range ($n = 8-11$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Ultrasonic Vocalisation:

Kruskal Wallis comparisons revealed a significant main effect on the duration of 22kHz ultrasonic vocalisation ($\chi^2(5) = 20.889$, $p < 0.001$) over the course of the 30-minute testing period. Further post-hoc analysis using Dunn-Bonferroni revealed that FC rats did not express a statistically significant increase in the duration of 22kHz ultrasonic vocalisation compared to NFC rats (FC VEH vs. NFC VEH, $p = 0.116$) over the course of the 30-minute testing period, despite the graphs showing trends towards increase. Intra-PrL MJN110 alone or in combination with AM251 had no effect on the duration of 22kHz ultrasonic vocalisation (Figure 4.7).

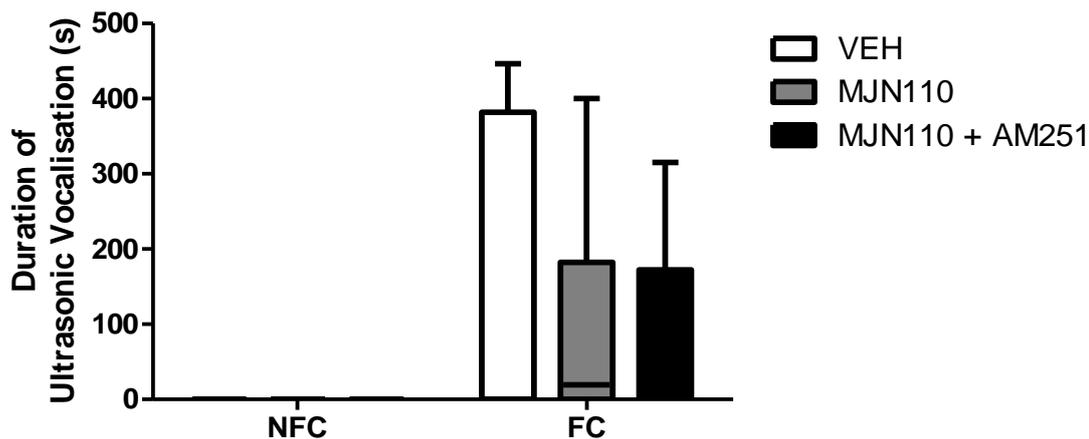


Figure 4.7: Effects of fear-conditioning and bilateral administration of MJN110 and MJN110+AM251 directly into the PrL on the duration ultrasonic vocalisation in rats over the full 30-minute testing period. All data are expressed as median with interquartile range (n=8-11 per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Analysis of the data subdivided into 10-minute time bins using Kruskal-Wallis revealed a significant between-group effect on the expression of 22kHz ultrasonic vocalisation at 0-10mins ($\chi^2(5)=23.169$, $p<0.001$) but not 10-20mins ($\chi^2(5)=5.093$, $p=0.405$) or 20-30mins ($\chi^2(5)=7.050$, $p=0.217$). Further post-hoc analysis using Dunn-Bonferroni revealed that FC rats did not express a statistically significant increase in 22kHz ultrasonic vocalisation (FC VEH vs. NFC VEH, $p=0.148$) compared to NFC rats over the course of the 30-minute testing period, despite the graphs showing a trend towards increase. Intra-PrL MJN110 alone or in combination with AM251 had no effect on the duration of 22kHz ultrasonic vocalisation (Figure 4.8).

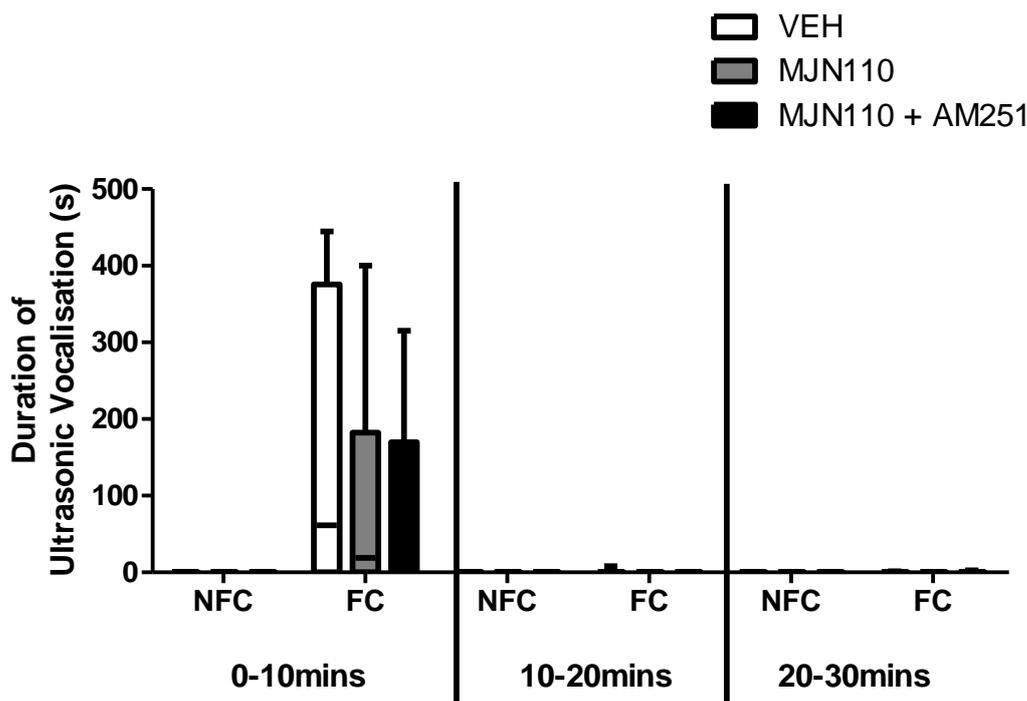


Figure 4.8: Effects of fear-conditioning and bilateral administration of MJN110 and MJN110+AM251 directly into the PrL on the duration of ultrasonic vocalisation in rats over the full 30-minute testing period subdivided into 10-minute time bins. All data are expressed as median with interquartile range (n=8-11 per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

4.3.4 Effects of fear-conditioning and MJN110 or MJN110+AM251 treatment on locomotor behaviours and defecation in formalin-treated rats

Two-way ANOVA revealed a significant main effect of fear-conditioning ($F(1,50)=6.895$, $p<0.05$) and treatment ($F(2,50)=8.846$, $p<0.05$) but not fear-conditioning*treatment ($F(2,50)=1.599$, $p=0.212$) on the distance moved over the course of the 30-minute testing period. Post-hoc analysis revealed that treatment with either MJN110 or MJN110+AM251 significantly decreased distance moved in fear-conditioned rats (FC VEH vs FC MJN110, $p<0.05$; FC VEH vs. FC MJN110+AM251, $p<0.01$). There was no significant drug effect in NFC rats (Table 4.2).

Two-way ANOVA revealed a significant main effect of fear-conditioning ($F(1,50)=9.180$, $p<0.01$) but not treatment ($F(2,50)=1.922$, $p=0.157$) or fear-conditioning*treatment

($F(2,50)=0.732$, $p=0.3486$) on the duration of walking over the 30-minute trial. Post-hoc analysis revealed no significant effects (Table 4.2).

Two-way ANOVA revealed a significant main effect of fear-conditioning ($F(1,50)=32.597$, $p<0.001$), but not treatment ($F(2,50)=2.126$, $p=0.130$) or fear-conditioning*treatment ($F(2,50)=2.649$, $p=0.081$) on the duration of rearing over the course of the testing period. Post-hoc analysis revealed that re-exposure of rats to the arena previously paired with footshock resulted in a significant increase in the duration of rearing (FC VEH vs. NFC VEH, $p<0.001$), an effect not seen in drug treated rats (FC MJN110 vs NFC MJN110, $p=0.740$; FC MJN110+AM251 vs. NFC MJN110+AM251, $p=0.570$). Treatment with either MJN110 alone or in combination with AM251 decreased the duration of rearing in FC rats (FC VEH vs FC MJN110, $p<0.01$; FC VEH vs FC MJN110+AM251, $p<0.01$). There was no significant drug effect in NFC rats (Table 4.2).

Two-way ANOVA revealed a significant main effect of fear-conditioning ($F(1,50)=8.890$, $p<0.01$) and fear-conditioning*treatment ($F(2,50)=3.821$, $p<0.05$) but not treatment ($F(2,50)=0.067$, $p=0.935$) on the duration of grooming. Post-hoc analysis revealed that re-exposure of rats to the arena previously paired with footshock resulted in a significant increase in the duration of grooming (FC VEH vs NFC VEH $p<0.01$), an effect not seen in drug treated rats (FC MJN110 vs NFC MJN110 $p=0.805$; FC MJN110+AM251 vs NFC MJN110+AM251 $p=0.999$). Treatment with MJN110 alone or in combination with AM251 decreased the amount of grooming in FC rats compared to FC VEH (FC VEH vs. FC MJN110; FC VEH vs FC MJN110+AM251, $p<0.05$). There were no significant drug effects in NFC rats (Table 4.2).

Kruskal Wallis comparisons revealed a significant effect ($\chi^2(5)=39.203$, $p<0.001$) for the amount of defecation in the arena (no. of pellets) over the course of the 30-minute testing period. Further post-hoc analysis by Dunn-Bonferroni revealed that FC rats (FC VEH vs. NFC VEH, $p<0.01$) exhibited a significant increase in defecation over the course of the 30-minute testing period compared to their NFC counterparts. Treatment with MJN110 alone or in combination with AM251 had no effect on the amount of defecation exhibited by rats (Table 4.2).

Treatment Group	Distance Moved (cm)	Walking (s)	Rearing (s)	Grooming (s)	Defecation (number of pellets)
NFC VEH	1875±120	57±11	3±1	21±7	2(1)
NFC MJN110	1499±144	38±8	2±1	30±7	2(2)
NFC MJN110+AM251	1469±133	40±5	4±2	41±12	2(2)
FC VEH	2366±186	81±12	65±20***	92±24***	7(3)**
FC MJN110	1718±113#	71±18	18±7#	45±20#	7(3)***
FC MJN110+AM251	1607±138#	58±9	22±8#	34±7#	5(2)*

Table 4.2: Effects of fear-conditioning and MJN110 or MJN110+AM251 on distance moved, walking, rearing, grooming and defecation in formalin treated rats. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. NFC; # $p < 0.05$ vs FC VEH (Tukey's/Dunn-Bonferroni respectively). All data are expressed as mean \pm SEM except defecation which is median (interquartile range) (n=8-11 per group). FC, fear-conditioned; NFC, non-fear-conditioned; VEH, vehicle.

4.3.5 Effect of fear-conditioning and vehicle or MJN110 on AEA, OEA, PEA or 2-AG levels in the mPFC of formalin-treated rats

Prelimbic Cortex:

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,33)=0.068$, $p=0.797$), treatment ($F(2,33)=0.752$, $p=0.481$), or fear-conditioning*treatment ($F(2,33)=0.616$, $p=0.547$) on the levels of AEA in the PrL (Figure 4.9).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,32)=0.352$, $p=0.558$), treatment ($F(2,32)=1.382$, $p=0.269$), or fear-conditioning*treatment ($F(2,32)=0.025$, $p=0.976$) on the levels of 2-AG in the PrL (Figure 4.9).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,32)=0.185$, $p=0.671$), treatment ($F(2,32)=1.299$, $p=0.290$), or fear-conditioning*treatment ($F(2,32)=0.476$, $p=0.626$) on the levels of PEA in the PrL (Figure 4.9).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,32)=0.406$, $p=0.530$), treatment ($F(2,32)=1.506$, $p=0.241$), or fear-conditioning*treatment ($F(2,32)=0.117$, $p=0.890$) on the levels of OEA in the PrL (Figure 4.9).

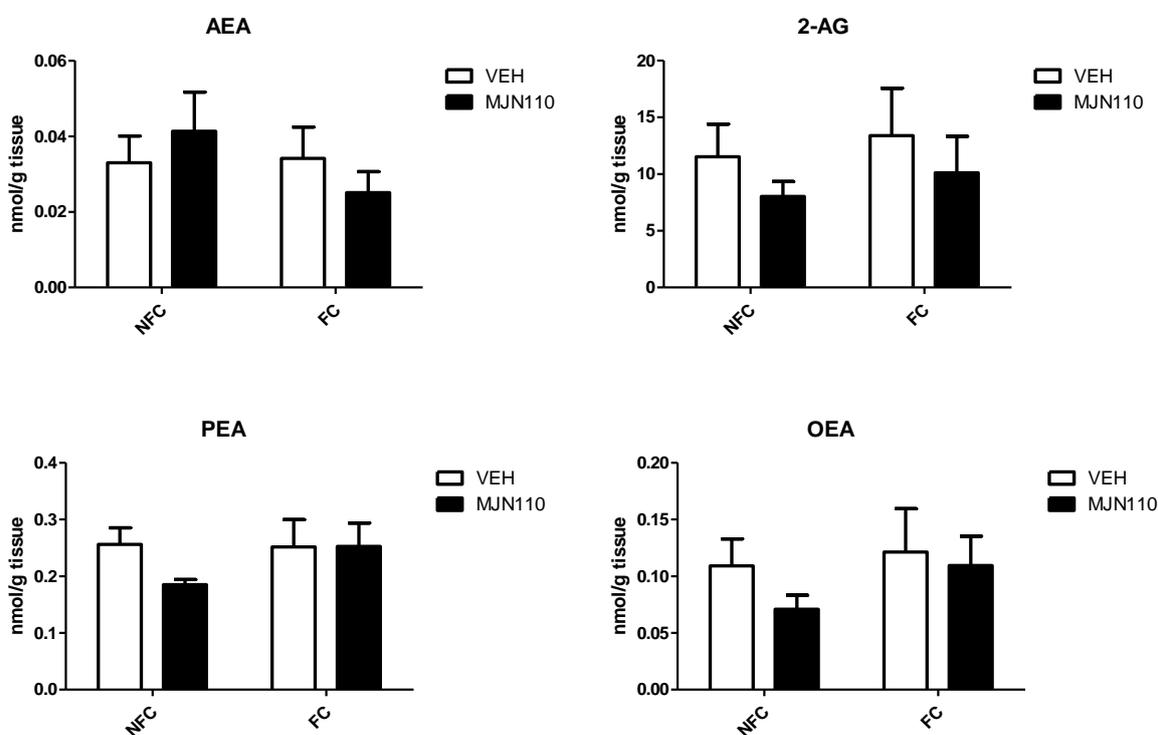


Figure 4.9: Effect of fear-conditioning and MJN110 on AEA, 2-AG, PEA or OEA levels in the PrL. All data are expressed as mean \pm SEM ($n=7-9$ per group); NFC-Non-fear-conditioned; FC-fear-conditioned; VEH-vehicle

Infralimbic Cortex:

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,34)=0.022$, $p=0.882$), treatment ($F(2,34)=0.018$, $p=0.982$), or fear-conditioning*treatment ($F(2,34)=0.004$, $p=0.996$) on the levels of AEA in the IL (Figure 4.10).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,32)=0.082$, $p=0.777$), treatment ($F(2,32)=0.813$, $p=0.455$), or fear-conditioning*treatment ($F(2,32)=0.612$, $p=0.545$) on the levels of 2-AG in the IL (Figure 4.10).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,34)=0.0044$, $p=0.836$), treatment ($F(2,34)=0.663$, $p=0.523$), or fear-conditioning*treatment ($F(2,34)=0.519$, $p=0.601$) on the levels of PEA in the IL (Figure 4.10).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,34)=0.031$, $p=0.862$), treatment ($F(2,34)=0.446$, $p=0.632$), or fear-conditioning*treatment ($F(2,34)=0.201$, $p=0.819$) on the levels of OEA in the IL (Figure 4.10).

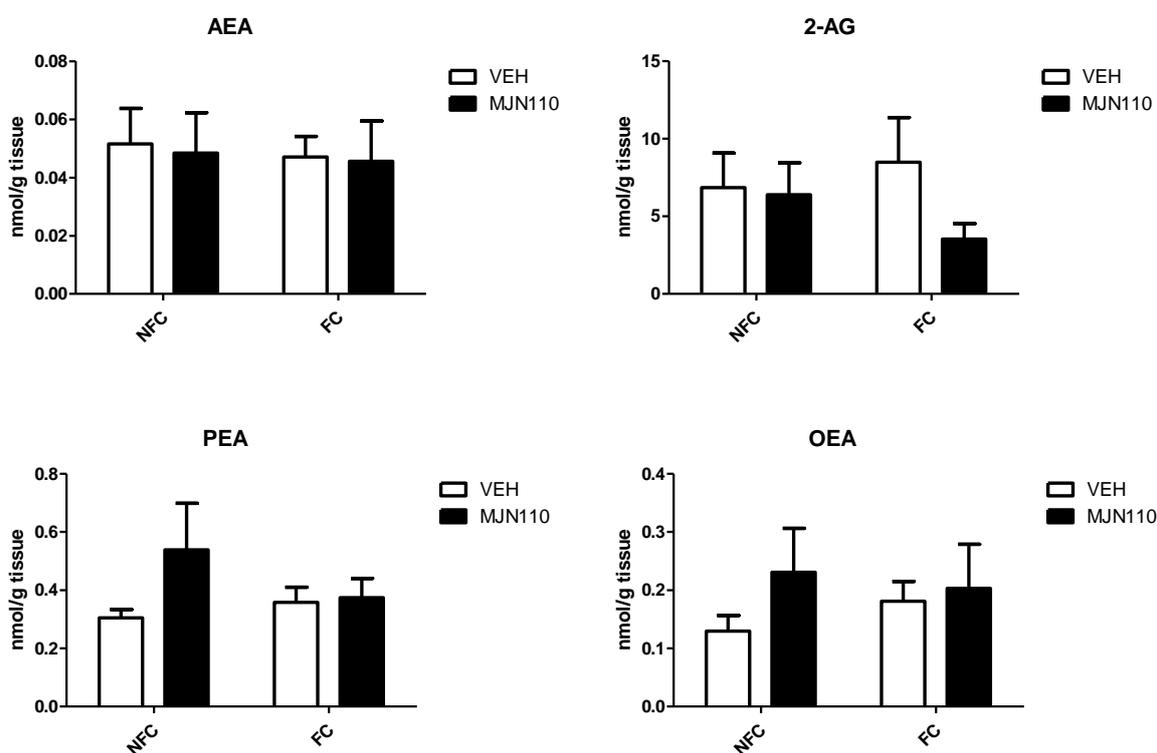


Figure 4.10: Effect of fear-conditioning and MJN110 on AEA, 2-AG, PEA or OEA levels in the IL. All data are expressed as mean \pm SEM ($n=7-10$ per group); NFC-Non-fear-conditioned; FC-fear-conditioned; VEH-vehicle

Anterior Cingulate Cortex:

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,34)=0.015$, $p=0.902$), treatment ($F(2,34)=0.017$, $p=0.983$), or fear-conditioning*treatment ($F(2,34)=0.514$, $p=0.603$) on the levels of AEA in the ACC (Figure 4.11).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,35)=0.155$, $p=0.697$), treatment ($F(2,35)=0.030$, $p=0.971$), or fear-conditioning*treatment ($F(2,35)=0.213$, $p=0.810$) on the levels of 2-AG in the ACC (Figure 4.11).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,35)=0.056$, $p=0.814$), treatment ($F(2,35)=0.430$, $p=0.655$), or fear-conditioning*treatment ($F(2,35)=0.670$, $p=0.519$) on the levels of PEA in the ACC (Figure 4.11).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,35)=0.113$, $p=0.739$), treatment ($F(2,35)=0.594$, $p=0.559$), or fear-conditioning*treatment ($F(2,35)=1.027$, $p=0.317$) on the levels of OEA in the ACC (Figure 4.11).

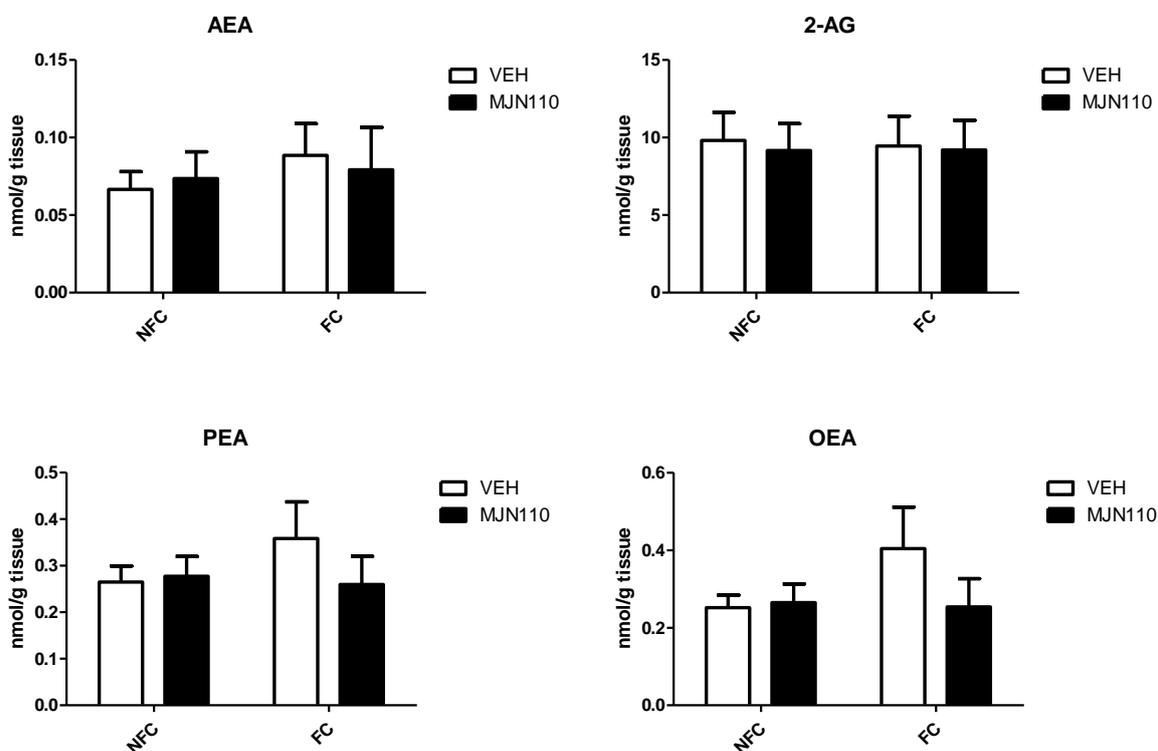


Figure 4.11: Effect of fear-conditioning and MJN110 on AEA, 2-AG, PEA or OEA levels in the ACC. All data are expressed as mean \pm SEM ($n=7-10$ per group); NFC-Non-fear-conditioned; FC-fear-conditioned; VEH-vehicle

4.4 Discussion

The data presented herein indicate for the first time that a MAGL substrate in the PrL modulates fear-pain interactions. In the absence of contextual fear conditioning, administration of the MAGL inhibitor alone or in combination with the CB₁ receptor antagonist AM251 had no effect on formalin-evoked nociceptive behaviour. Re-exposure of vehicle-treated rats to a context previously paired with foot shock significantly reduced formalin-evoked nociceptive behaviour (i.e. induced FCA) and increased contextually induced freezing in the presence of formalin-evoked nociceptive tone. Microinjection of the MAGL inhibitor MJN110 into the PrL attenuated the expression of FCA, increasing formalin-evoked nociceptive behaviour, an effect unopposed upon co-administration with AM251. MJN110 alone or in combination with AM251 had no effect on the expression of conditioned fear in the presence of nociceptive tone. Treatment with MJN110 alone or in combination with AM251 decreased the distance moved in FC rats compared with FC VEH treated rats. Fear-conditioning also significantly increased the duration of rearing and grooming in VEH rats, an effect blocked by with MJN110 alone or in combination with AM251. Mass spectrometry revealed that neither FC nor intra-PrL administration of MJN110 were associated with alterations of endocannabinoids or related n-acylethanolamines in the PrL, IL or ACC at the end of the 30-minute testing period. These data demonstrate that a MAGL substrate in the PrL is an important neural substrate regulating the expression of FCA.

In the present study, VEH-treated FC rats exhibited a reduction in formalin-evoked nociceptive behaviour upon re-exposure to a context previously paired with an aversive stimulus (i.e. footshock), confirming the expression of FCA. This finding is consistent with the two earlier chapters and a number of previous studies, within our own group and others, investigating FCA (Fanselow, 1984, Finn et al., 2004, Olango et al., 2012, Rea et al., 2013, Roche et al., 2007, Helmstetter, 1992, Helmstetter and Bellgowan, 1993, Rea et al., 2014a, Rea et al., 2009).

MAGL inhibition via MJN110 had no effect on the expression of fear-related behaviours in the presence of nociceptive tone but attenuated FCA and increased the expression of formalin-evoked nociceptive behaviour in FC rats. This effect was not blocked upon co-administration with the CB₁ receptor antagonist AM251. These findings together suggest that in the PrL, the expression of FCA is suppressed by a MAGL substrate, and provide further evidence that the PrL is an important neural substrate for endocannabinoid-related FCA (Rea et al., 2018), in addition to the ventral hippocampus (Ford et al., 2011), dIPAG (Olango et al., 2012) and the BLA (Rea et al., 2013, Roche et al., 2007) and IL (Rea et al., 2018).

The effects of MJN110 alone were not blocked by co-administration of AM251, suggesting that a MAGL substrate modulates expression of FCA via a non-CB₁ receptor target. As discussed in chapter 3, other non-CB₁ targets of 2-AG could include CB₂ (Gonsiorek et al., 2000, Sugiura et al., 2000), GABA_A (Sigel et al., 2011), PPARs (Bouaboula et al., 2005), Adenosine A₃ (Lane et al., 2010), TRPV1 (Yusaku et al., 2008) and GPR55 (Ryberg et al., 2007).

Our group has previously investigated a role for FAAH substrates in the PrL in FCA. FAAH inhibition in the PrL via URB597 prolonged, while CB₁ receptor blockade in the PrL via AM251 attenuated FCA (Rea et al., 2018), effects not seen upon co-administration. The present results extend these previous findings and further implicate an important role for the endocannabinoid system in the PrL in FCA. These results also indicate opposing roles for both FAAH and MAGL substrates in the PrL, as FAAH inhibition prolonged, while MAGL inhibition attenuated, the expression of FCA. Similarly, the enhancement of FCA via FAAH inhibition likely acted via a CB₁ receptor mechanism, as the effect was blocked by co-administration with AM251, while the attenuation of FCA via MAGL inhibition was not affected by CB₁ receptor blockade indicating that FAAH and MAGL substrates may have different mechanisms of action, acting via CB₁ and non-CB₁ receptor targets respectively.

Rea et al., found that intra-PrL administration of URB597 and AM251 modulated the expression of FCA without having any effect on contextually induced fear behaviour, demonstrating that pain-related behaviour in fear-conditioned animals can be altered independently of the level of fear being expressed in these animals in the presence of nociceptive tone (Rea et al., 2018). This result is comparable with the results herein which suggest that intra-PrL administration of MJN110 alone or in combination with AM251 attenuated FCA while having no effect on fear-related behaviours (contextually induced freezing and 22kHz ultrasonic vocalisation) indicating that the effects of pain and fear were dissociable. These data corroborate with the results presented in chapter 3 for the IL study, along with previous studies from our own research group (Finn et al., 2004, Rea et al., 2013, Roche et al., 2010, Roche et al., 2007) and those of others (Helmstetter and Fanselow, 1987, Kinscheck et al., 1984), demonstrating that FCA can be altered independently of the expression of fear-related behaviour in these animals in the presence of nociceptive tone.

Rea et al., demonstrated an opposing effect of FAAH substrates in the IL and PrL with FAAH inhibition attenuating and enhancing FCA respectively (Rea et al., 2018). In contrast, the

present study has established that a MAGL substrate may play a similar role in both the IL and PrL on the expression of FCA as MAGL inhibition attenuates FCA regardless of the region it is injected into, see chapter 3. Furthermore, Rea et al., demonstrated differential effects of FAAH inhibition on the expression of contextually induced fear in the presence of nociceptive tone whereby intra-IL URB597 attenuated, while intra-PrL URB597 had no effect on, the expression of freezing in the presence of nociceptive tone. In contrast, the present studies have established that MAGL inhibition in either region, have no effect on the expression of contextually induced fear behaviours in the presence of nociceptive tone. These data indicate that depending on the region (IL or PrL) FAAH and MAGL substrates may have differential or common effects on the expression of FCA and fear-related behaviours in the presence of tone.

Like the IL, intra-PrL administration of MJN110 decreased the distance moved in FC rats compared to VEH. This effect was not blocked by co-administration with AM251, indicating a role for a non-CB₁ receptor target. The drug had no effect on the distance moved exhibited by NFC rats so the decrease in distance moved in FC rats was likely not due to an impairment in locomotor activity. As in chapter 3, decreased distance moved in these rats may relate to the increase in formalin-evoked nociceptive behaviour seen upon treatment with either MJN110 or MJN110+AM251. It is possible the rats spend more time immobile with their formalin-injected paw elevated or spend more time in the one spot licking and biting the injected paw. Fear-conditioning increased the duration of rearing in VEH-treated rats, an effect blocked by intra-PrL MJN110 or MJN110+AM251. As in chapter 3, it is possible that this drug is affecting active (rearing) but not passive (freezing) behaviour in fear-conditioned rats or this drug exerts a possible anxiolytic effect by acting on a non-CB₁ receptor. Fear-conditioning also increased the duration of grooming in FC rats, an effect blocked by MJN110 and MJN110+AM251, again indicating a possible anxiolytic effect of this drug acting on a non-CB₁ receptor. In humans and rodents excessive grooming occurs in stressful situations and can be linked to neuropsychiatric disorders in humans such as OCD (Kalueff et al., 2016, Ahmari et al., 2013). In our study, the increase in the duration of grooming upon fear-conditioning correlates with an increase in fear-related behaviour. Therefore, the increased grooming could imply an increase in stress/anxiety in FC rats. Attenuation by a MAGL inhibitor could imply an anxiolytic role for a MAGL substrate in the PrL, as MJN110 decreases the stress-related grooming. MAGL inhibition in the IL and PrL of rats seems to have similar effects on locomotor and exploratory behaviours, suggesting a regionally common effect of a MAGL substrate.

Though intra-PrL administration of the MAGL inhibitor MJN110 affected FCA in the present study, I did not find any significant between-group differences in tissue levels of endocannabinoids or related *N*-acylethanolamines in the PrL, IL or ACC. I expected to see an increase in 2-AG levels as seen in previous studies with the same dose of MJN110 (Sticht et al., 2016). However, these findings corroborate the previous IL study (chapter 3) where there was also no increase in 2-AG levels. Unlike the IL, intra-PrL administration did not increase the levels of AEA in the PrL and may indicate that MJN110 has a different mechanism of action in the IL and PrL. As mentioned in chapter 3, it is possible that the effects of MJN110 are mediated by elevation of the MAGL substrate 2-oleoylglycerol. As in chapters 2 and 3, the brains are removed and frozen 45-minute post microinjection in our study, it is possible that 2-AG levels were increased in the PrL during this experiment, but the time point at which we measured the levels was not optimal to reveal any increased 2-AG levels. Future studies could measure the levels of endocannabinoids or related *N*-acylethanolamines at earlier time points, such as 30-minutes post microinjection / 15 minutes after re-exposure to the arena previously paired with footshock when the expression of both FCA and fear-related behaviours is maximal. This would aid in understanding the mechanism of action by which 2-AG exerts its effects.

In conclusion, the present study provides new evidence to support a role for a MAGL substrate, in the PrL in the expression of FCA via a non CB₁-related receptor. Intra-MJN110 had no effect on the levels of 2-AG in the mPFC. Thus, the precise mechanisms underlying the behavioural effects observed in this study require further investigation. The evidence presented in this study corroborate the work presented in chapter 3 indicating a similar effect for a MAGL substrate in the IL and PrL. Taken together the data provide further evidence that the PrL is a key neural substrate for endocannabinoid-mediated regulation of FCA.

Chapter 5 : The role of 2-AG in the anterior cingulate cortex in formalin-evoked nociceptive behaviour, fear-conditioned analgesia and conditioned fear in the presence of nociceptive tone in rats

5.1 Introduction

The ACC is a subregion of the mPFC believed to be homologous with Brodmann's areas 24, 25, 32 and 33 of the human brain (Stevens et al., 2011). The ACC has numerous projections, including but not limited to the other subregions of the mPFC (IL and PrL), midcingulate cortex, insular cortex, amygdala, hypothalamic nuclei, hippocampus, PAG, brainstem centres, orbitofrontal cortex, striatum, raphe nucleus, thalamus and temporoparietal junction (Stevens et al., 2011, Wang et al., 2014a). The ACC is located at the front of the cingulate cortex surrounding the frontal part of the corpus callosum. It is located dorsally to the IL and PrL. The ACC is ideally located within the brain to mediate inputs from brain regions and exert top down modulating effects. The large number of projections of the ACC reflect its many functions including, autonomic functions, reward and punishment, memory, pain modulation and anxiety and fear (Stevens et al., 2011).

As stated in chapter 2, the ACC plays a key role in the regulation of fear-related behaviours. Dopamine and 5HT, both known to play a key role in neuropsychiatric disorders, have been shown to be key neurotransmitters in the ACC in an observational fear learning paradigm in mice (Kim et al., 2014). The amygdala is a widely studied region with important functional roles in the regulation, expression and extinction of fear-related behaviours (Duvarci and Pare, 2014, Marek et al., 2013). The amygdala shares connections with the ACC that, when measured via MRI following fear acquisition training in humans, exhibit enhanced functional connectivity, indicating an important role for this pathway in memory consolidation of fear conditioning (Feng et al., 2014). The ACC may also play a key role in the development of PTSD, a mental disorder that develops after a person is exposed to a traumatic event. Studies have shown a correlation between thickness, volume, pattern and activation of the ACC and PTSD symptomology (Jatzko et al., 2013, Shu et al., 2014, Dickie et al., 2013). The above information, combined with the ACC projections to/from the amygdala, hippocampus, nucleus

accumbens, IL and PrL (regions with a role in fear), suggest a possible role for the ACC in the top-down regulation of fear-related processes.

The ACC is also a key cortical region for pain perception (Zhuo, 2014, Apkarian et al., 2005, Vogt, 2005) and has been implicated in the affective component of pain (Treede et al., 1999). Neural activity is increased in the ACC of humans, primates, rodents and rabbits in response to a noxious stimulus (Sikes and Vogt, 1992, Hutchison et al., 1999, Koyama et al., 1998, Yamamura et al., 1996). Early lesion studies indicated a pro-nociceptive role for the ACC in chronic pain in humans (Foltz and White, 1962, Hurt and Ballantine, 1974). In humans, PET imaging has revealed that ACC activation is positively correlated with the degree and magnitude of unpleasantness in response to a painful stimulus (Rainville et al., 1997). The ACC shares connections with numerous brain regions that play a key role in pain processing including the PAG, amygdala, IL and PrL. It may act as a relay point and function to regulate the top-down modulation of the descending inhibitory pain pathway.

The role of the endocannabinoid system in pain and fear is well established and has been discussed in detail in the introduction of this thesis. The role of the endocannabinoid system in the ACC in pain and fear is not as well established. Our own laboratory has found that intra-ACC administration of PEA attenuated formalin-evoked nociceptive behaviour in rats, an effect attenuated by the CB₁ receptor antagonist AM251. Furthermore, antagonism of CB₁, TRPV1 or PPAR α and γ in the ACC also attenuated formalin-evoked nociceptive behaviour (Okine et al., 2014). These results suggest a clear role for the endocannabinoid system in the ACC in inflammatory pain-related behaviour. In mice, cannabinoid-induced G-protein coupling is decreased in the ACC of mice 10 days following chronic constriction injury, indicating a role for the endocannabinoid system in this region in neuropathic pain (Hoot et al., 2010). In humans, THC reduced the reported unpleasantness of hyperalgesia induced by capsaicin in healthy human volunteers. fMRI revealed that the anti-hyperalgesic effect of THC in these volunteers was associated with decreased activity in the ACC volunteers (Lee et al., 2013). Similarly, cannabidiol injected directly into the ACC reduced mechanical allodynia in a rat model of surgical incision pain (Genaro et al., 2017). As mentioned in the previous chapters, our laboratory has investigated the effects of the FAAH inhibitor URB597 and CB₁ antagonist AM251 on FCA in the mPFC. Despite having clear effects in the IL and PrL (attenuating and enhancing FCA respectively) neither drug had any effect in the ACC (chapter 2). However, the role of 2-AG in the ACC has not yet been investigated and is the main aim of this chapter.

As previously mentioned in chapters 3 and 4, 2-AG plays a role in the regulation of fear, anxiety and pain (Bluett et al., 2017, Busquets-Garcia et al., 2011, Sciolino et al., 2011, Sumislawski et al., 2011, Rea et al., 2014a, Wilkerson et al., 2017, Llorente-Berzal et al., 2015, Hartley et al., 2016). However, no studies to date have investigated the role of 2-AG and MAGL in the ACC in conditioned fear in the presence of nociceptive tone, formalin-evoked nociceptive behaviour and FCA. Until recently CB₂ receptors were largely unexplored supraspinally and because of this the role of CB₂ in the brain in pain regulation remains to be fully elucidated. CB₂ receptors are expressed in the PFC (den Boon et al., 2012) but the role of CB₂ in the PFC in pain and fear has yet to be established.

This chapter will assess the role of 2-AG in the ACC in formalin-evoked nociceptive behaviour, FCA and conditioned fear in the presence of nociceptive tone with the following hypothesis: 2-AG-CB₁ signalling in the ACC will have no effect on the expression of formalin-evoked nociceptive behaviour, conditioned fear or FCA. Therefore, the overall aims of the study described in this chapter were:

- To determine the role of 2-AG in the ACC in formalin-evoked nociceptive behaviour, FCA and expression of fear behaviour in the presence of formalin-evoked nociceptive tone. This was achieved by examining the effects of intra-ACC administration of the MAGL inhibitor, MJN110 alone or in combination with the CB₁ receptor antagonist/inverse agonist.
- To examine the role of 2-AG and CB₂ in the ACC in formalin-evoked nociceptive behaviour, expression of fear behaviour in the presence of formalin-evoked nociceptive tone and expression of FCA. This was achieved by examining the effects of intra-ACC administration of the MAGL inhibitor, MJN110 or the CB₂ antagonist AM630 alone or in combination.
- To examine if behavioural changes are associated with alterations in endocannabinoid levels in the ACC and surrounding mPFC regions using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).
- To determine whether CB₂ mRNA is expressed in the mPFC of rats
- To determine whether fear conditioning has any effect on CB₁ and CB₂ mRNA expression in the ACC.

5.2 Materials and Methods

This chapter presents the results of two experiments; experiment 1 and experiment 2.

5.2.1 Animals:

Experiment 1:

Male Lister-Hooded rats (262–310g on day of behavioural testing; Charles River, Margate, Kent, UK) were used. Animals were housed 3 per cage before surgery and singly thereafter in plastic bottomed cages (45 x 20 x 20cm) with wood shavings as bedding. They were maintained at a constant temperature ($22 \pm 2^\circ\text{C}$) under standard lighting conditions (12:12 hour light–dark, lights on from 0700-1900h). Experiments were carried out during the light phase between 0700 and 1700h. Food and water were available *ad libitum*. Subjects were randomly assigned to experimental groups and the sequence of testing was randomized throughout the experiment. The experimental procedures were approved by the Animal Care and Research Ethics Committee, National University of Ireland, Galway, and the work carried out under license from the Health Products Regulatory Authority and in compliance with the European Communities Council directive 86/609 and 2010/63 and conformed to the ARRIVE guidelines.

Experiment 2:

Male Lister-Hooded rats (266–326g on day of behavioural testing; Charles River, Margate, Kent, UK) were used. All other details were identical to experiment 1.

5.2.2 Cannulae implantation:

Experiment 1 and 2:

See Chapter 2 section 2.2.2 for details on cannula implantation via stereotaxic surgery.

Briefly, stainless steel guide cannulae (Plastics One Inc., Roanoke, Virginia, USA) were stereotaxically implanted bilaterally 1mm above the right and left ACC (AP + 1mm relative to bregma, ML \pm 1.3mm at a 12° angle, DV – 1.3mm from dura, toothbar set at -3mm) (Paxinos and Watson, 1998).

Following surgery, animals were singly housed and a single daily dose of enrofloxacin (2.5mg/kg, s.c., Baytril; Bayer Ltd., Dublin, Ireland) was administered for the following four days. A minimum of 6 days was allotted to allow rats to recover before any experimentation commenced. During this period, the rats were handled, stylets checked, and their body weight and general health monitored.

5.2.3 Chemicals and drug preparation:

Experiment 1 and 2:

Formalin (37% formaldehyde solution), DMSO (dimethyl sulfoxide, 100%), AM251 (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide), MJN110 (2,5-dioxopyrrolidin-1-yl 4-(bis(4-chlorophenyl)methyl)piperazine-1-carboxylate), and AM630 (6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone, AM630, Iodopravadoline) were purchased from Sigma-Aldrich, Dublin, Ireland.

On test days, solutions of 2µg/µl MJN110 (MAGL inhibitor), 5mM AM630 (CB₂ receptor antagonist), 2µg/µl MJN110+2mM AM251 (CB₁ receptor antagonist/inverse agonist), or 2µg/µl MJN110+5mM AM630 were prepared using stock solutions and 100% DMSO vehicle. A solution of 2.5% formalin (Sigma-Aldrich, Dublin, Ireland) was prepared from a 37% stock solution diluted with 0.9% sterile saline.

These doses of AM251, MJN110 and AM630 were chosen based on previous work carried out by our laboratory (including that described chapters 3 and 4 of this thesis) and evidence from the literature (Lisboa et al., 2010, Rea et al., 2018, Freitas et al., 2013, Sticht et al., 2016, Wills et al., 2016, Almeida-Santos et al., 2013, Stern et al., 2017).

5.2.4 Experimental procedure:

Experiment 1:

The experimental procedure was identical to that described in chapter 2 section 2.2.4.

On test days, rats received intra-ACC microinjection (0.3µl per side) of either vehicle (VEH; 100% DMSO), the MAGL inhibitor MJN110 (2µg/µl) or the CB₁ receptor antagonist MJN110+AM251 (MJN110: 2µg/µl; AM251:2mM). A group receiving AM251 alone was not

included because the effects of intra-ACC administration of AM251 alone on the same behavioural parameters in an identical experimental design were investigated in Chapter 2 of this thesis.

After 30-minutes in the arena, rats were removed and euthanised by decapitation. Brains and spinal cords were rapidly removed within a 2-minute timeframe, snap-frozen on dry ice and stored at -80°C. After decapitation, 0.3µl of 2% fast green dye (dissolved in DMSO) was also injected into the left and right ACC for post-mortem confirmation of microinjection sites during cryo-sectioning.

This design resulted in 6 experimental groups (Starting n=12 per group for surgery; final n=8-11 per group for data analysis) as illustrated in Table 5.1.

Group	Conditioning	Formalin i.pl.	Drug/Vehicle	N
1	FC	Formalin	100% DMSO	9
2	NFC	Formalin	100% DMSO	8
3	FC	Formalin	MJN110	10
4	NFC	Formalin	MJN110	9
5	FC	Formalin	MJN110+AM251	11
6	NFC	Formalin	MJN110+AM251	10

Table 5.1: Summary of experimental groups in experiment 1; i.pl., intraplantar; FC, fear-conditioned; NFC, non – fear-conditioned

Experiment 2:

The experimental procedure was identical to that described in chapter 2 section 2.2.4.

On test days, rats received intra-ACC microinjection (0.3µl per side) of either vehicle (VEH; 100% DMSO), the MAGL inhibitor MJN110 (2µg/µl), 5mM AM630 (CB₂ receptor antagonist), or 2µg/µl MJN110 + 5mM AM630.

After 30-minutes in the arena, rats were removed and euthanised by decapitation. Brains and spinal cords were rapidly removed within a 2-minute timeframe, snap-frozen on dry ice and stored at -80°C. After decapitation, 0.3µl of 2% fast green dye (dissolved in DMSO) was also

injected into the left and right ACC for post-mortem confirmation of microinjection sites during cryo-sectioning.

This design resulted in 8 experimental groups (Starting n=10 per group for surgery; final n=8-10 per group for data analysis) as illustrated in Table 5.2.

Group	Conditioning	Formalin i.pl.	Drug/Vehicle	N
1	FC	Formalin	100% DMSO	9
2	NFC	Formalin	100% DMSO	8
3	FC	Formalin	MJN110	8
4	NFC	Formalin	MJN110	8
5	FC	Formalin	AM630	10
6	NFC	Formalin	AM630	10
7	FC	Formalin	MJN110+AM630	10
8	NFC	Formalin	MJN110+AM630	8

Table 5.2. Summary of experimental groups in experiment 2; i.pl., intraplantar; FC, fear-conditioned; NFC, non – fear-conditioned

5.2.5 Brain removal:

See chapter 2 section 2.2.5 for both experiment 1 and 2.

5.2.6 Behavioural analysis:

See chapter 2 section 2.2.6 for both experiment 1 and experiment 2.

5.2.7 Histological verification of intracerebral injection sites:

See chapter 2 section 2.2.7 for both experiment 1 and experiment 2.

5.2.8 Tissue isolation by Palkovits punch:

See chapter 3 section 3.2.8 for both experiment 1 and experiment 2.

5.2.9 Measurement of levels of endocannabinoids in the medial prefrontal cortex:

See chapter 2 section 2.2.9 with the same amendments as chapter 3 section 3.2.9 for both experiment 1 and experiment 2.

5.2.10 Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was carried out as described previously (Burke et al., 2014, Kerr et al., 2012, Rea et al., 2014).

RNA was extracted from the pellets collected from mPFC tissue (IL: 1.45mg±0.2mg; PrL 1.8mg±0.25mg; ACC: 2mg±0.3mg) following processing for mass spectrometry using the Machery-Nagel NucleoSpin ® RNA II extraction kit (Nucleospin RNA II, Technopath, Ireland), according to the manufacturer's instructions. Tissue was homogenised in 350µl of lysis buffer (RA1), containing 1% β-mercaptoethanol (Sigma, Ireland) for 3-5s using an automated homogenizer (Polytron tissue disrupter, Ultra-Turrax, Germany). Homogenates were kept on ice until transferred to a Nucleospin filter (violet ring) and centrifuged at 11000g for 1 min. The lysates were then treated with 350µl of 70% molecular grade ethanol (Sigma, Ireland) and transferred to a Nucleospin RNA spin column II (light blue ring) and centrifuged at 11000 g for 30s to bind the RNA. The membrane column was then desalted using 350µl membrane desalting buffer (MDB). RNA samples were then treated with 10µl DNase and left for 15 minutes at room temperature to remove DNA from the sample. Samples were then serially washed using washing buffers (200µl RA2, 600µl RA3 and 250µl RA3) and RNA was eluted in 20µl of RNAase-free water (Sigma, Ireland).

Nanodrop technology (ND-1000, Nanodrop, Labtech International, Ringmer, UK) was used to measure the quantity, purity and quality of the RNA. RNA quantity was determined by measuring optical density (OD) at 260 nm. RNA quality was determined by measuring the ratio OD260/OD280 where a ratio of approximately 1.8-2.1 was deemed indicative of pure RNA. All RNA samples with a ratio >1.6 deemed acceptable. Samples were equalised to the same concentration of RNA (10ng/µl) using RNase free water (Sigma, Ireland). Equalised samples were then stored at -80°C until reverse transcribed.

Equal amounts of total RNA (10ng/µl) were reverse transcribed into cDNA as follows. Two master mixes were made up as shown below in Tables 5.3 and 5.4, all reagents were obtained from (Biosciences, Dublin, Ireland). 10µl of normalised RNA from each sample was added to a newly labelled PCR tube where 2µl of master mix 1 was added to each tube. The mixture was

then heated to 65°C for 5 minutes in a thermocycler (MJ Research, INC, USA) and quickly chilled on ice. The contents of the tube were collected by brief centrifugation. 7µl of master mix 2 was added to each tube, mixed gently and incubated at 37°C for 2 minutes on the thermocycler. 1µl of superscriptIII reverse transcriptase was added to each sample and gently missed. Samples were left to incubate at room temperature for 10-minutes and then for 50 minutes at 50°C. Samples were then inactivated by heated at 70°C for 15 minutes. Finally cDNA samples were diluted (1:4) using RNAase-free water and stored at -20°C.

Reagents	Per Sample
Random Primers (250ng)	1µl
10mm dNTP mix	1µl
Total	2µl

Table 5.3: Master mix 1 for cDNA synthesis

Reagents	Per Sample
5X First Strand Buffer	4µl
0.1M DTT	2µl
RNase Out	1µl
Total	7µl

Table 5.4: Master mix 2 for cDNA synthesis

Single stranded cDNA products were then analysed by real-time quantitative PCR using the Applied Biosystems StepOne Plus Real Time PCR System (Bio-Sciences, Dublin, Ireland). Taqman gene expression assays (Bio-Sciences, Dublin, Ireland) containing forward and reverse primers and a FAM-labelled MGB Taqman probe were used (Bio-Sciences, Dublin, Ireland). Assay IDs for the genes examined were as follows for rat CNR2 (CB₂) (Rn03993699) or CNR1 (CB₁) (Rn02758689) and VIC-labelled GAPDH (Rn_4308313) as the house keeping gene and endogenous control. A reaction mixture was prepared and stored on ice. This consisted of 0.5µl target primers (Bio-Sciences, Dublin, Ireland) 0.5µl of the reference gene GAPDH (multiplex version), 5µl TaqMan master mix, 1.5µl of RNA free water and 2.5µl of sample cDNA to give 10µl total per sample. Samples were pipetted in duplicate, (10µl per well

total volume) into an optical 96 well plate. Negative controls were included in all assays, containing the master mix cDNA replaced with RNA free water. Plates were then covered with adhesive covers and spun at 1000g for 1 minute to ensure complete mixing. The plate was then placed in StepOnePlus™ real time PCR machine (Bio-Sciences, Dublin, Ireland). StepOnePlus™ cycling conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of (95°C for 15 sec/60°C for 1 min). See Figure 5.1 for an example of the amplification plots for *CNR1* (*CB₁*), *CNR2* (*CB₂*) and *GAPDH*.

Amplification plots and copy threshold (Ct) values were examined using Applied Biosystems 7500 System SDS Software 1.3.1 and exported to Microsoft Excel for final analysis. The 2- $\Delta\Delta$ CT method was used to determine gene expression. This method is used to assess relative gene expression by comparing gene expression of experimental samples to control samples, allowing determination of the fold change in mRNA expression between experimental groups. This method involves 3 steps: (1) Normalisation to endogenous control (*GAPDH*) where Δ Ct is determined: Δ Ct = Ct Target gene - Ct Endogenous control gene; (2) Normalisation to control sample where $\Delta\Delta$ Ct is determined: $\Delta\Delta$ Ct = Δ Ct Sample - average Δ Ct of Control group; and (3) where the fold difference is given by $2^{-\Delta\Delta$ Ct}. The $2^{-\Delta\Delta$ Ct values for each sample were then expressed as a percentage of the average of the $2^{-\Delta\Delta$ Ct values for the control group (i.e. NFC VEH). In this manner the percentage increase or decrease in mRNA expression between experimental groups was determined.

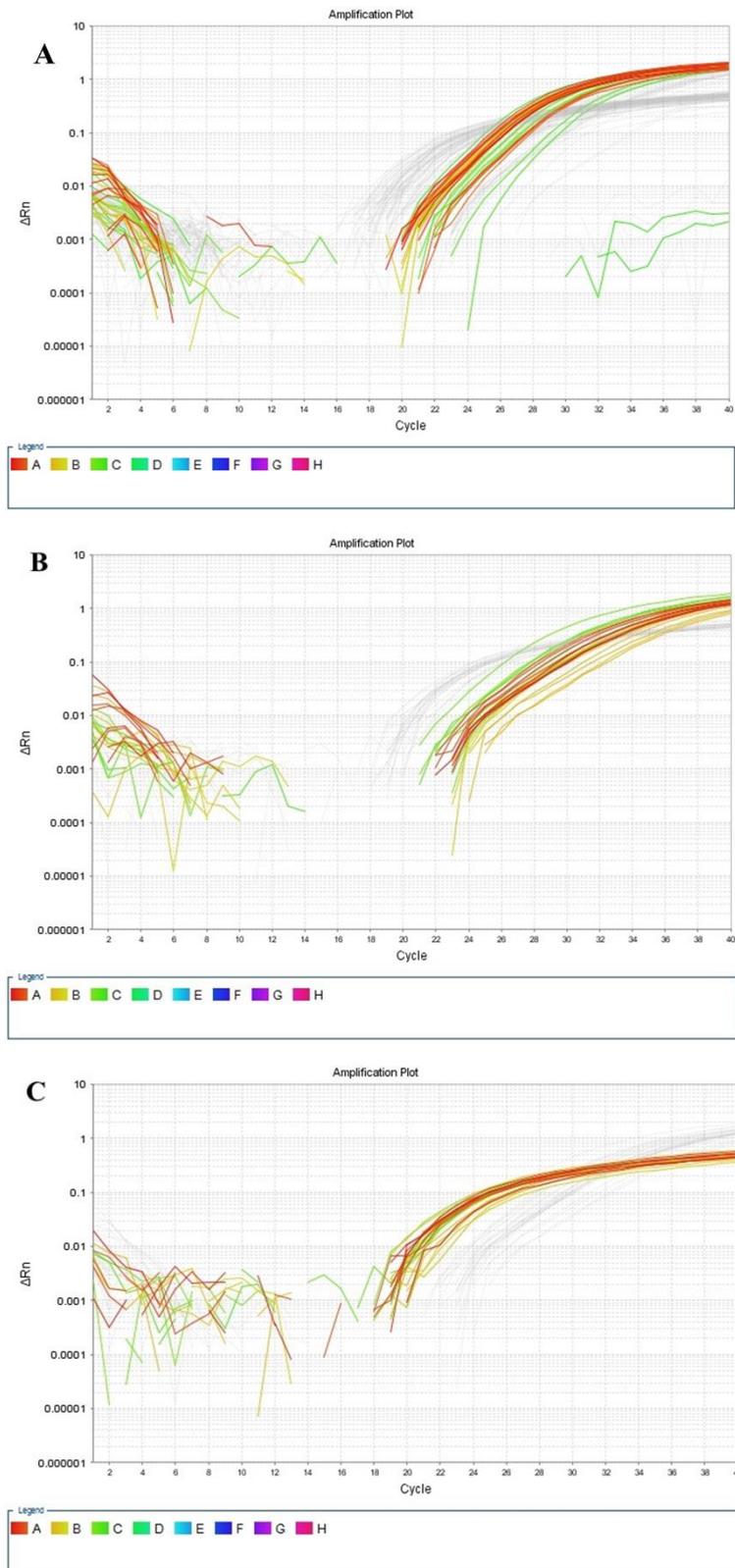


Figure 5.1: Example amplification plots for (A) *CNR1* (CB_1) (B) *CNR2* (CB_2) (C) *GAPDH*

5.2.11 Statistical analysis:

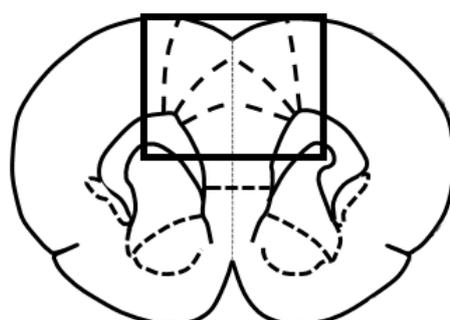
The IBM SPSS statistical software package (SPSS v23.0 for Microsoft Windows; Chicago, IL, USA) was used to analyse all data. Normality and homogeneity of variance were assessed using Shapiro-Wilk's and Levene's test, respectively. All data except the duration of freezing, 22kHz ultrasonic vocalisation and defecation were found to be parametric. Behavioural data and mass spectrometry data were analysed by 2-way ANOVA, with fear-conditioning and drug treatment as factors. For ACC qRT-PCR data an independent samples, two-tailed t-test was run. Time course behavioural data were analysed by 2-way repeated measures ANOVA with time as the within-subjects factor and fear-conditioning and drug treatment as the between-subjects factors. Sphericity was tested using Mauchly's Test for Sphericity. If sphericity was violated a Greenhouse-Geisser correction was used. Post-hoc pairwise comparisons were made with Tukey's test when appropriate. Non-parametric data were analysed using Kruskal Wallis analysis of variance and post-hoc analysis performed using Dunns-Bonferroni pairwise comparisons when appropriate. For repeated measures non-parametric data, Kruskal Wallis was performed followed by Dunn-Bonferroni post-hoc where appropriate. Results are expressed as group means \pm SEM or median with interquartile range and min/max.

5.3 Results

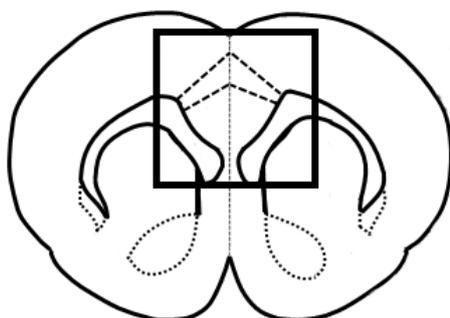
5.3.1 Results of experiment 1:

5.3.1.1 Histological verification of microinjection sites

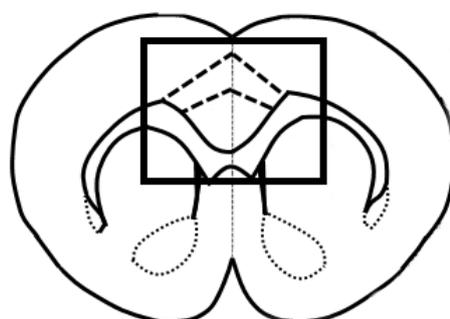
86% of the injections were within the borders of the ACC (Figure 5.2) with the remaining 14% placed just above the ACC in the motor cortex (M2) or lateral to the ACC in the corpus callosum. The data analysed were derived only from rats in which bilateral injections were correctly positioned in the ACC.



+ 2.2 mm from Bregma



+ 1.7 mm from Bregma



+ 1.6 mm from Bregma

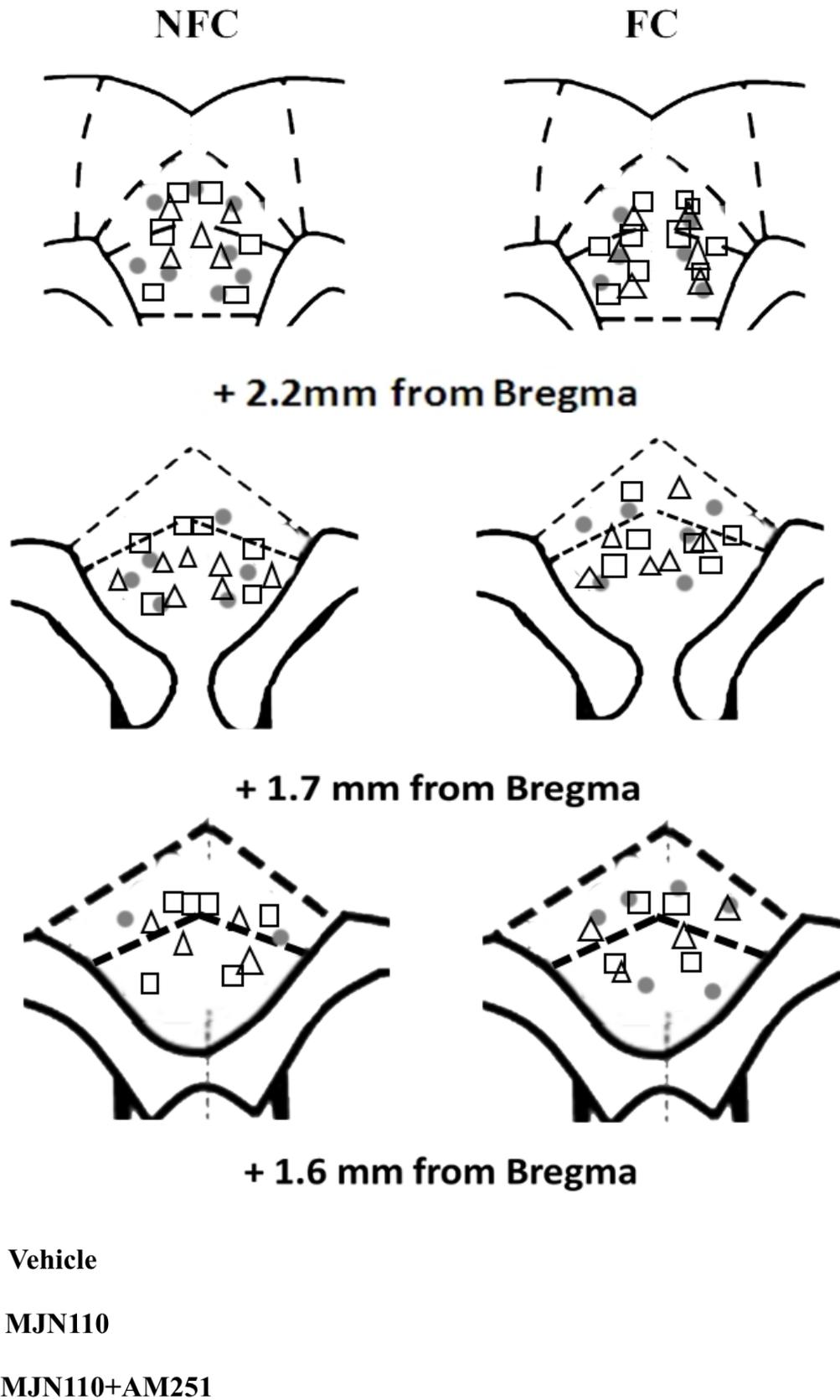


Figure 5.2: Histological verification of injector site location. Adapted from Paxinos & Watson (1998). NFC: Non-fear-conditioned; FC: Fear-conditioned

5.3.1.2 Effects of intra-ACC administration of MJN110, or MJN110+AM251 on formalin-evoked nociceptive behaviour and FCA

Intra-plantar injection of formalin induced a robust nociceptive response in the form of elevation, licking, biting and shaking of the injected right hind-paw. Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,51)=0.121$, $p=0.992$), treatment ($F(2,51)=0.442$, $p=0.645$) or fear-conditioning*treatment ($F(2,51)=0.799$, $p=0.456$) on the change in hind paw diameter pre- versus post-formalin injection (Figure 5.3).

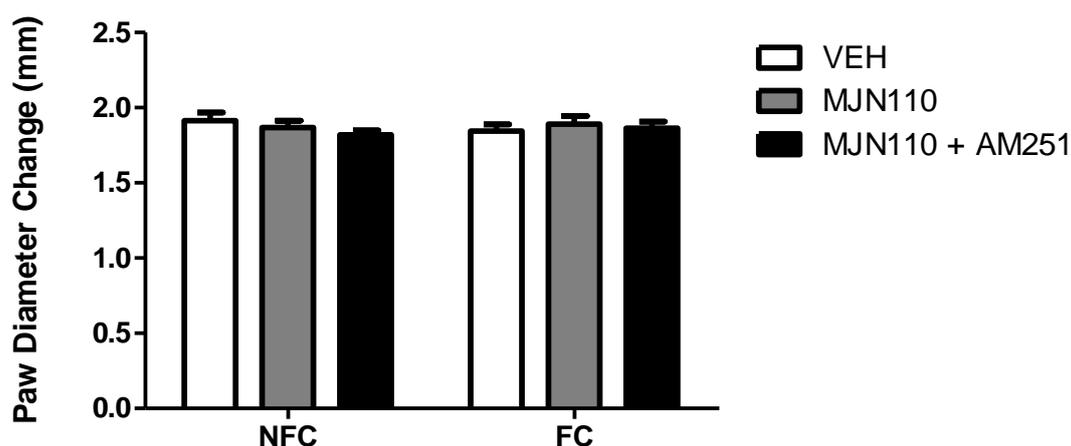


Figure 5.3: Effects of fear-conditioning and bilateral administration of MJN110 or MJN110+AM251 directly into the ACC on paw diameter change (mm) pre and post formalin injection (oedema). All data are expressed as mean \pm SEM ($n=8-11$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Two-way ANOVA revealed a significant effect of fear-conditioning ($F(1,51)=76.225$, $p<0.001$), treatment ($F(2,51)=34.957$, $p<0.001$) and fear-conditioning*treatment ($F(2,51)=10.960$, $p<0.001$) on CPS. Further post-hoc analysis revealed that re-exposure of rats to the arena previously paired with footshock resulted in a significant reduction in formalin-evoked nociceptive behaviour, confirming the expression of FCA (FC VEH vs. NFC VEH, $p<0.001$). Intra-ACC administration of MJN110 or MJN110+AM251 significantly attenuated the expression of FCA over the course of the 30-minute trial (FC VEH vs. FC MJN110 or FC MJN110+AM251, $p<0.001$). There was no significant drug effect in NFC rats (Figure 5.4).

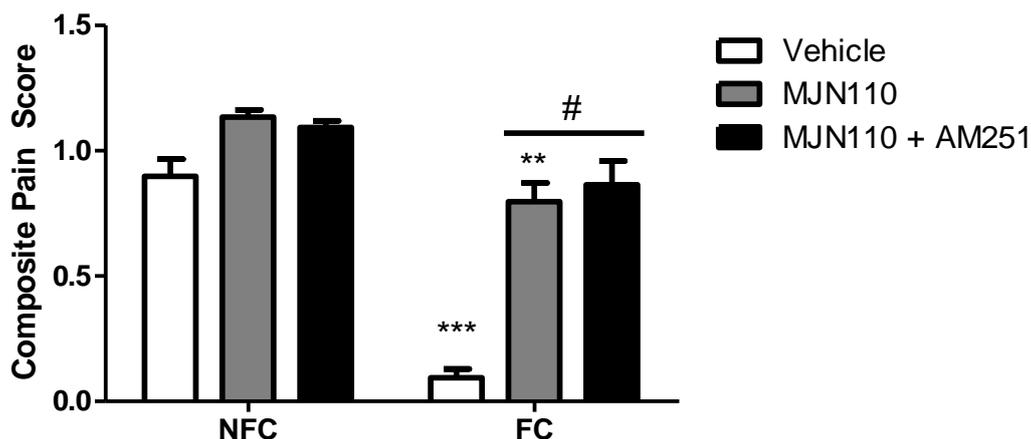


Figure 5.4: Effects of fear-conditioning and bilateral administration of MJN110 and MJN110+AM251 directly into the ACC on formalin-evoked nociceptive behaviour in rats over the full 30-minute testing period. ** $p < 0.01$, *** $p < 0.001$ FC vs. NFC; # $p < 0.001$ vs FC VEH (Tukey's). All data are expressed as mean \pm SEM ($n = 8-11$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Temporal analysis of the data subdivided into 10-minute time bins and using 2-way repeated measures ANOVA revealed a significant main effect of fear-conditioning ($F(1,51) = 76.224$, $p < 0.001$), treatment ($F(2,51) = 34.955$, $p < 0.001$) and fear-conditioning*treatment ($F(2,51) = 10.959$, $p < 0.001$) on CPS. Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated ($\chi^2(2) = 15.252$, $p < 0.001$) and therefore a Greenhouse-Geisser correction was used. There was a significant main effect of time ($F(1.58,80.77) = 22.406$, $p < 0.001$), time*fear-conditioning ($F(1.58,80.77) = 7.815$, $p < 0.001$) and time*fear-conditioning*treatment ($F(3.17,80.77) = 4.796$, $p < 0.01$) but not time*treatment ($F(3.17,80.77) = 0.472$, $p = 0.713$) on CPS over the course of the testing period. Further post-hoc analysis revealed a significant reduction in formalin-evoked nociceptive behaviour in FC VEH treated rats for the entire 30-minute testing period (FC VEH vs. NFC VEH, 0-10mins, 10-20mins, 20-30mins, $p < 0.001$) confirming the expression of FCA throughout the trial. Intra-ACC administration of MJN110, alone or in combination with AM251, significantly attenuated the expression of FCA, in the final 20 minutes of the trial (FC VEH vs. FC MJN110 or FC MJN110+AM251, 10-20mins, 20-30mins, $p < 0.001$). MJN110+AM251 also attenuated the expression of FCA in the first 10-minutes of the testing period (FC VEH vs. FC

MJN110+AM251, $p < 0.001$) with similar trends in the first 10-minutes of the trial for MJN110. There was no significant drug effect in non-fear-conditioned animals (Figure 5.5).

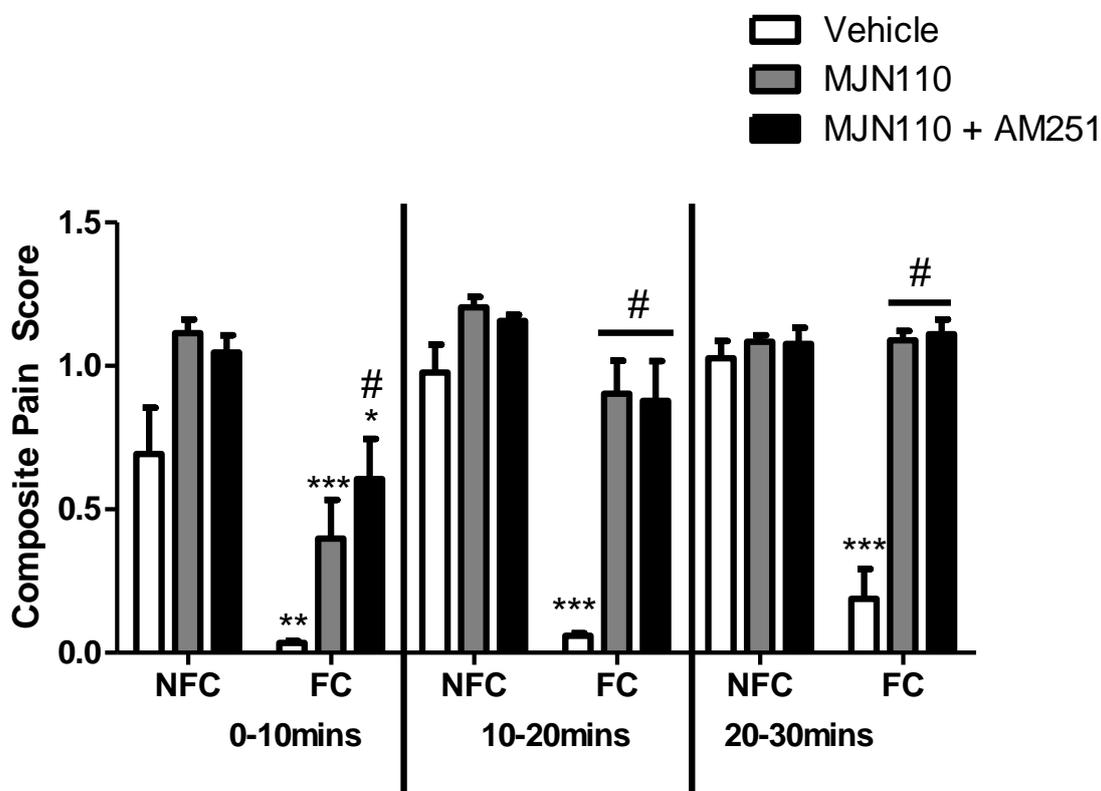


Figure 5.5: Effects of fear-conditioning and bilateral administration of MJN110 and MJN110+AM251 directly into the ACC on formalin-evoked nociceptive behaviour in rats over the full 30-minute testing period subdivided into 10-minute time bins. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ FC vs. NFC; # $p < 0.001$ vs FC VEH (Tukey's). All data are expressed as mean \pm SEM ($n = 8-11$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

5.3.1.3 Effects of intra-ACC administration of MJN110 or MJN110+AM251 on expression of conditioned fear behaviour in the presence of formalin-evoked nociceptive tone

Duration of Freezing:

Kruskal Wallis analysis revealed a significant main effect on the duration of freezing ($\chi^2(5) = 17.444$, $p < 0.01$) over the course of the 30-minute testing period. Further post-hoc analysis using Dunn-Bonferroni revealed that re-exposure to an arena previously paired with

footshock did not increase freezing behaviour in a statistically significant manner despite the graphs showing trends towards an increase (Figure 5.6).

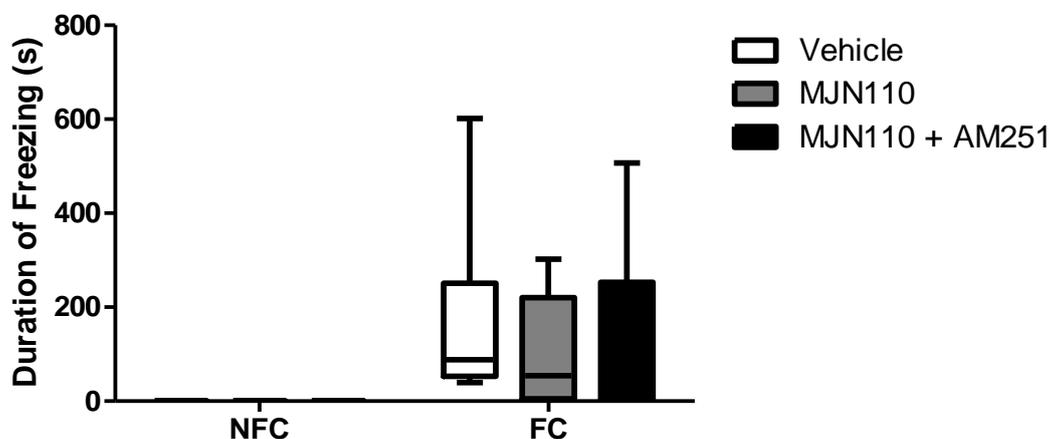


Figure 5.6: Effects of fear-conditioning and bilateral administration of MJN110 and MJN110+AM251 directly into the ACC on duration of freezing behaviour in rats over the full 30-minute testing period. All data are expressed as median with interquartile range (n=8-11 per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Analysis of the data subdivided into 10-minute time bins using Kruskal-Wallis revealed a significant between-group effect on the expression of freezing at 0-10mins ($\chi^2(5)=40.143$, $p<0.001$), 10-20mins ($\chi^2(5)=15.710$, $p<0.001$) but not 20-30mins ($\chi^2(5)=10.857$, $p=0.055$). Post-hoc analysis using the Dunn-Bonferroni test revealed that fear-conditioned rats receiving intra-ACC VEH displayed significantly increased freezing behaviour in the first 10-minutes of the trial, compared with NFC VEH treated counterparts (FC VEH vs. NFC VEH; 0-10mins, $p<0.01$). Intra-ACC MJN110 alone or in combination with AM251 had no effect on the duration of freezing (Figure 5.7).

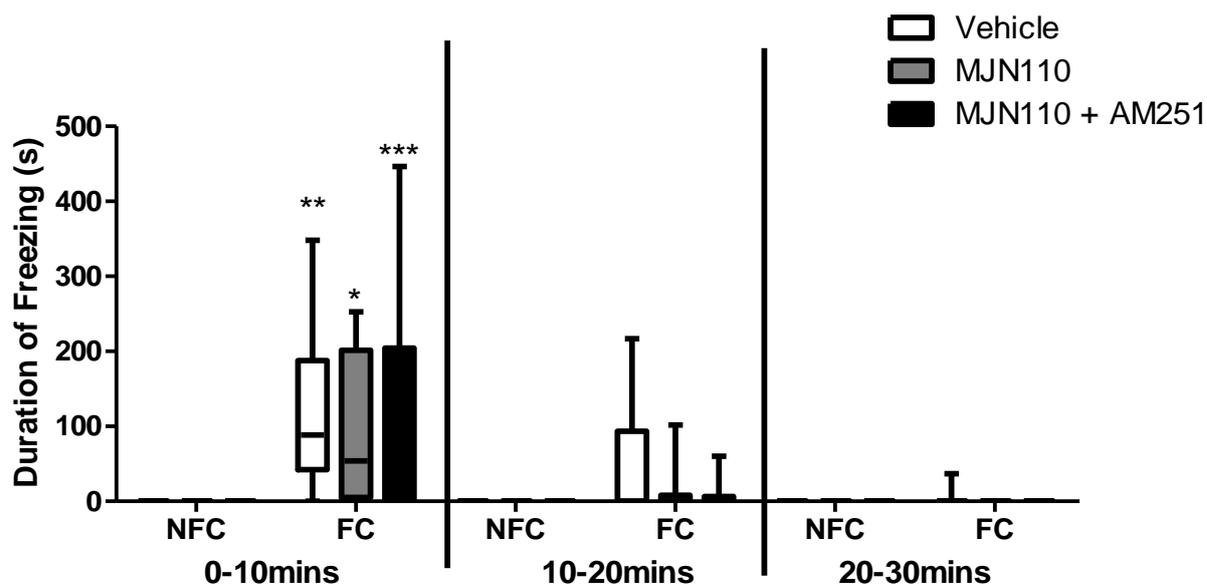


Figure 5.7: Effects of fear-conditioning and bilateral administration of MJN110 and MJN110+AM251 directly into the ACC on duration of freezing behaviour in rats over the full 30-minute testing period subdivided into 10-minute time bins. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs NFC (Dunn-Bonferroni). All data are expressed as median with interquartile range ($n = 8-11$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Ultrasonic Vocalisation:

Kruskal Wallis analysis revealed a significant main effect on the duration of 22kHz ultrasonic vocalization ($\chi^2(5) = 40.652$, $p < 0.001$) over the course of the 30-minute testing period. Further post-hoc analysis using Dunn-Bonferroni revealed that FC rats expressed a significantly higher duration of 22kHz ultrasonic vocalization (FC VEH vs. NFC VEH, $p < 0.001$) compared to their NFC counterparts over the course of the 30-minute testing period. Intra-ACC MJN110 alone or in combination with AM251 had no significant effect on the duration of 22kHz ultrasonic vocalisation (Figure 5.8).

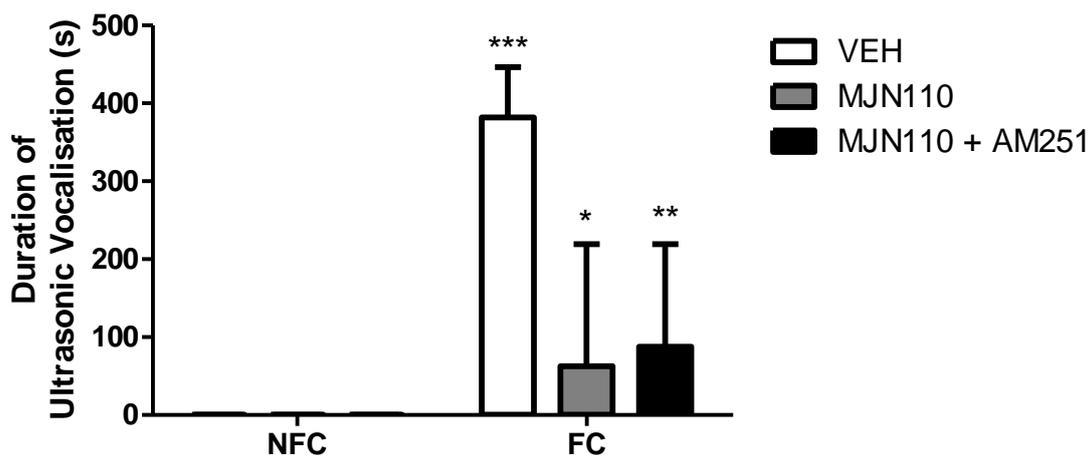


Figure 5.8: Effects of fear-conditioning and bilateral administration of MJN110 and MJN110+AM251 directly into the ACC on duration of ultrasonic vocalisation in rats over the full 30-minute testing period. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs NFC; (Dunn-Bonferroni). All data are expressed as median with interquartile range ($n = 8-11$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Analysis of the data subdivided into 10-minute time bins using Kruskal-Wallis revealed a significant between-group effect on the expression of 22kHz ultrasonic vocalisation at 0-10mins ($\chi^2(5) = 19.042$, $p < 0.01$) but not 10-20mins ($\chi^2(5) = 10.311$, $p = 0.067$) or 20-30mins ($\chi^2(5) = 5.333$, $p = 0.377$). Further post-hoc analysis using Dunn-Bonferroni revealed that re-exposure to an arena previously paired with footshock did not increase the duration of 22kHz ultrasonic vocalisation in a statistically significant manner despite the graphs showing trends towards an increase (Figure 5.9).

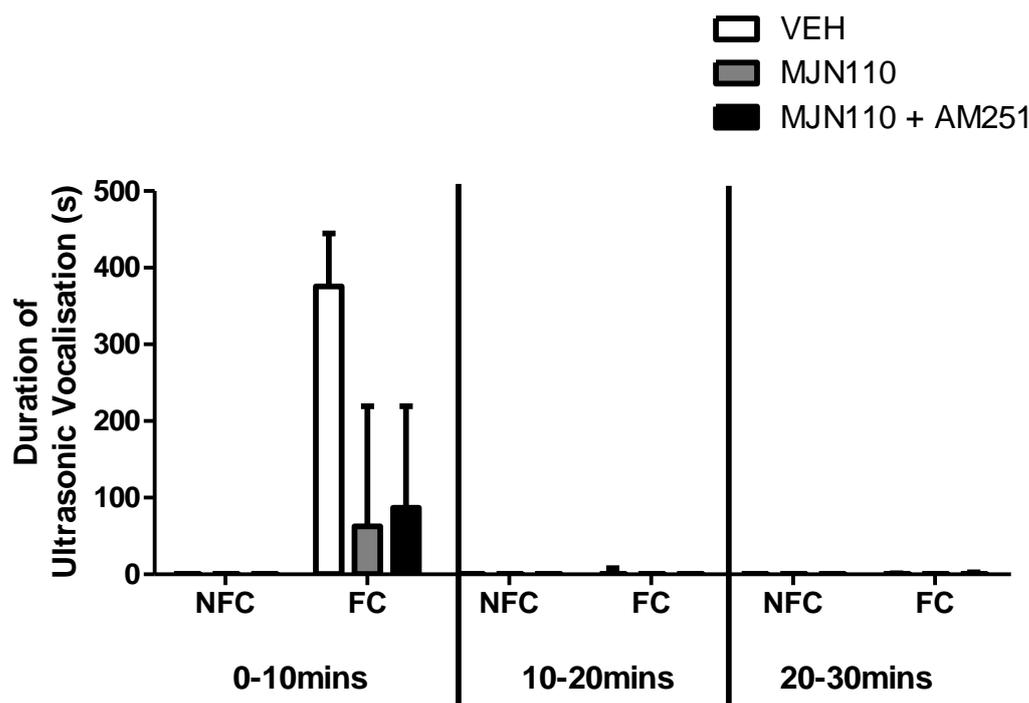


Figure 5.9: Effects of fear-conditioning and bilateral administration of MJN110 and MJN110+AM251 directly into the ACC on formalin-evoked nociceptive behaviour in rats over the full 30-minute testing period subdivided into 10-minute time bins. All data are expressed as median with interquartile range (n=8-11 per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

5.3.1.4 Effects of fear-conditioning and MJN110 or MJN110+AM251 treatment on locomotor behaviours and defecation in formalin-treated rats

Two-way ANOVA revealed a significant effect of treatment ($F(2,51)=5.224$, $p=0.009$) and fear-conditioning*treatment ($F(2,51)=5.930$, $p=0.005$) but not fear-conditioning alone ($F(1,51)=0.517$, $p=0.475$) on the distance moved over the 30-minute testing period. Further post-hoc analysis revealed a significant increase in the amount of distance moved in FC VEH treated rats compared to NFC VEH (FC VEH vs. NFC VEH, $p<0.05$). Treatment with either MJN110 or MJN110+AM251 also attenuated this effect in FC rats compared to FC VEH (FC VEH vs. FC MJN110; FC VEH vs. FC MJN110+AM251, $p<0.001$). There were no significant drug effects in NFC treated rats (Table 5.5).

Two-way ANOVA revealed a significant effect of fear-conditioning*treatment ($F(2,51)=3.341$, $p<0.05$) but not fear-conditioning ($F(1,51)=0.091$, $p=0.764$) or treatment

($F(2,51)=0.949$, $p=394$) alone on the duration of walking over the 30-minute testing period. Further post-hoc analysis revealed no significant effects in either FC or NFC treated rats (Table 5.5).

Two-way ANOVA revealed a significant effect of fear-conditioning ($F(1,51)=31.733$, $p<0.001$), treatment ($F(2,51)=21.438$, $p<0.001$) and fear-conditioning*treatment ($F(2,51)=9.239$, $p<0.001$) on the duration of rearing over the 30-minute testing period. Further post-hoc analysis revealed a significant increase in the duration of rearing in FC VEH treated rats compared to NFC VEH (FC VEH vs. NFC VEH, $p<0.001$). This effect was not seen in rats treated with either MJN110 alone or in combination with AM251 (FC MJN110 vs. NFC MJN110, $p=0.210$; FC MJN110+AM251 vs. NFC MJN110+AM251 $p=0.967$). Treatment with either MJN110 or MJN110+AM251 also decreased the duration of rearing in FC rats compared to FC VEH (FC VEH vs. FC MJN110; FC VEH vs FC MJN110+AM251, $p<0.001$). There were no significant drug effects in NFC treated rats (Table 5.5).

Two-way ANOVA revealed a significant effect of fear-conditioning ($F(1,51)=13.594$, $p<0.001$) and treatment ($F(2,51)=4.126$, $p<0.05$) but not fear-conditioning*treatment ($F(2,51)=1.676$, $p=0.197$) on the duration of grooming over the 30-minute testing period. Further post-hoc analysis revealed a significant increase in the duration of grooming in FC VEH treated rats compared to NFC VEH (FC VEH vs. NFC VEH, $p<0.05$). This effect was not seen upon administration with either MJN110 alone or in combination with AM251 (FC MJN110 vs. NFC MJN110, $p=0.886$; FC MJN110+AM251 vs. NFC MJN110+AM251 $p=0.492$). Treatment with MJN110 or MJN110+AM251 also decreased the duration of grooming in FC rats compared to FC VEH (FC VEH vs. FC MJN110; FC VEH vs FC MJN110+AM251, $p<0.05$). There were no significant drug effects in NFC treated rats (Table 5.5).

Kruskal Wallis comparisons from k independent samples revealed a significant effect ($\chi^2(5)=30.312$, $p<0.001$) for the amount of defecation in the arena (no. of pellets) over the 30-minute testing period. Further post-hoc analysis by Dunn-Bonferroni revealed that FC rats (FC-VEH vs. NFC VEH, $p<0.05$) exhibited a significant increase in defecation over the course of the 30-minute testing period compared to their NFC counterparts. Treatment with MJN110 alone or in combination with AM251 had no effect on the amount of defecation exhibited by rats (Table 5.5).

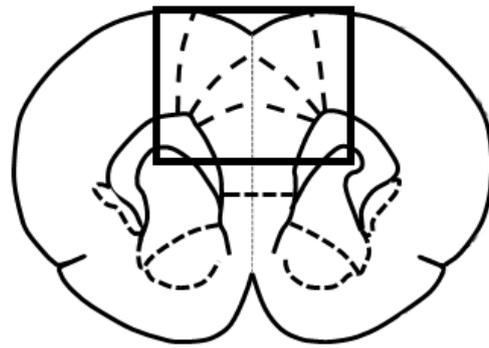
Treatment Group	Distance Moved (cm)	Walking (s)	Rearing (s)	Grooming (s)	Defecation (number of pellets)
NFC VEH	1810±102	76±9	28±9	54±13	1(1)
NFC MJN110	1870±157	87±15	5±2	50±13	2(2)
NFC MJN110+AM251	1808±127	95±20	10±4	35±10	2(2)
FC VEH	2541±250*	116±11	118±18***	123±13*	5(3)*
FC MJN110	1595±166#	68±11	36±10#	71±18#	6(3)*
FC MJN110+AM251	1629±96#	66±8	21±7#	67±12#	5(3)*

Table 5.5: Effects of fear-conditioning and MJN110 or MJN110+AM251 on distance moved, walking, rearing duration, grooming and defecation in formalin treated rats. * $p < 0.05$, *** $p < 0.001$ vs. NFC; # $p < 0.05$ vs FC VEH (Tukey's/Dunn-Bonferroni). All data are expressed as mean \pm SEM except for defecation which is median with interquartile range (n=8-11, per group) FC, fear-conditioned; NFC, non-fear-conditioned; VEH, vehicle.

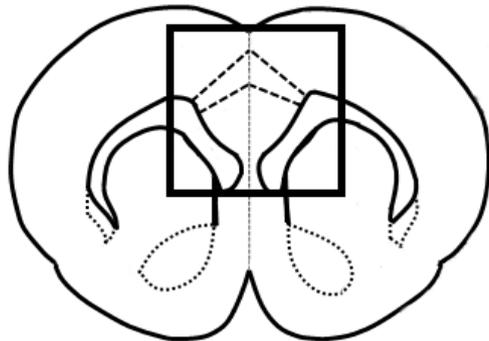
5.3.2 Results of experiment 2:

5.3.2.1 Histological verification of microinjection sites

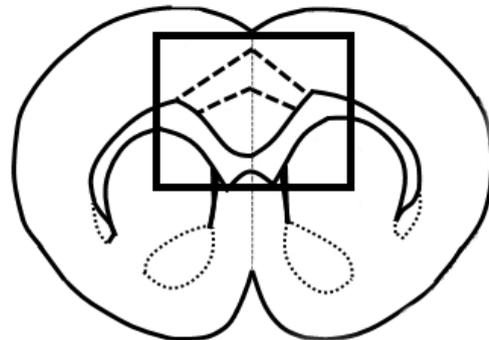
89% of the injections were within the borders of the ACC (Figure 5.10) with the remaining 11% placed just above the ACC in the motor cortex (M2) or lateral to the ACC in the corpus callosum. The data analysed were derived only from rats in which bilateral injections were correctly positioned in the ACC.



+ 2.2 mm from Bregma



+ 1.7 mm from Bregma



+ 1.6 mm from Bregma

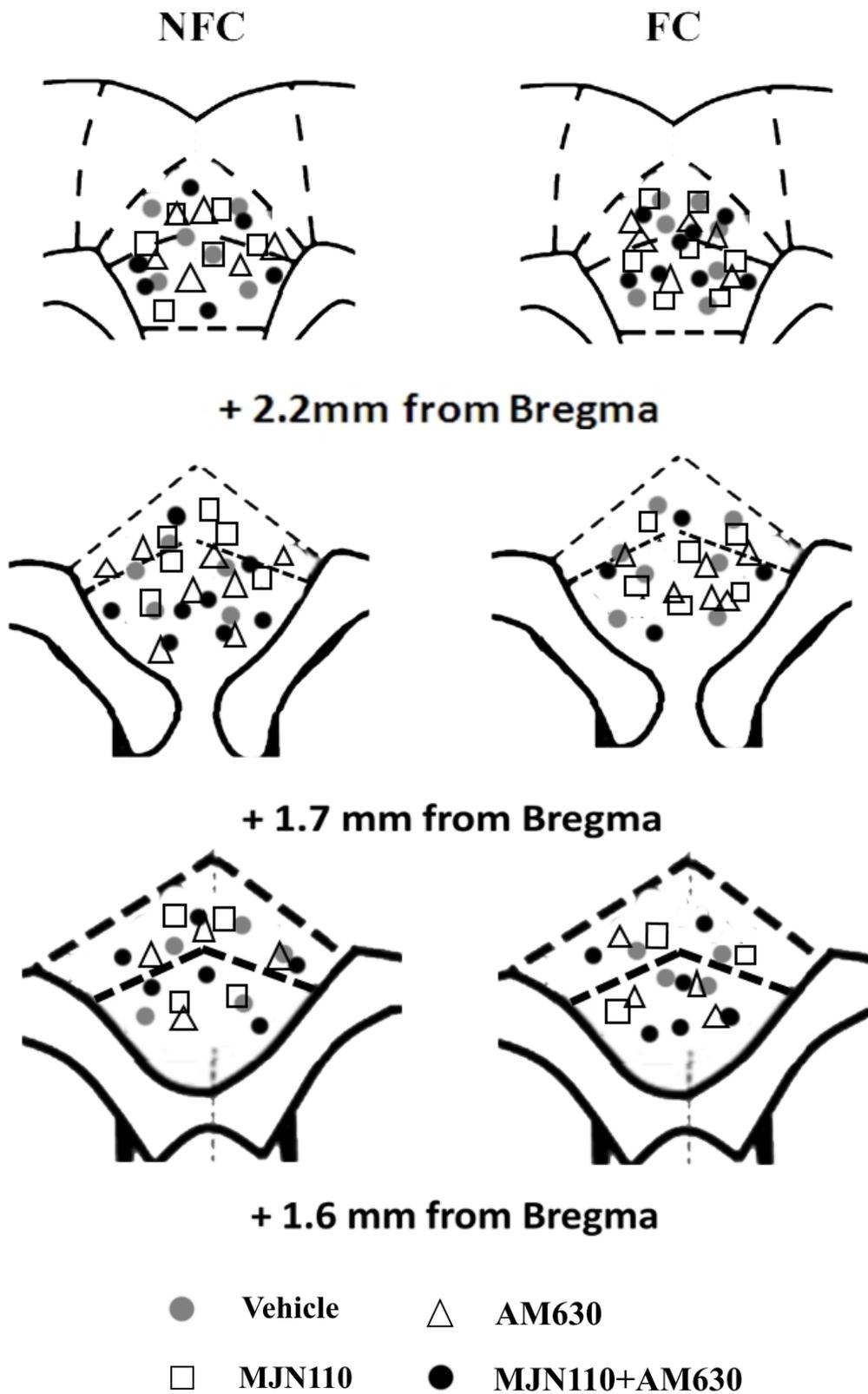


Figure 5.10: Histological verification of injector site location. Adapted from Paxinos & Watson (1998). NFC: Non-fear-conditioned; FC: Fear-conditioned

5.3.2.2 Effects of intra-ACC administration of MJN110, AM630, or MJN110+AM630 on formalin-evoked nociceptive behaviour and FCA

Intra-plantar injection of formalin induced a robust nociceptive response in the form of elevation, licking, biting and shaking of the injected right hind-paw. Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,71)=0.165$, $p=0.686$), treatment ($F(3,71)=0.396$, $p=0.756$) or fear-conditioning*treatment ($F(3,71)=1.436$, $p=0.241$) on the change in hind paw diameter pre- versus post-formalin injection (Figure 5.11).

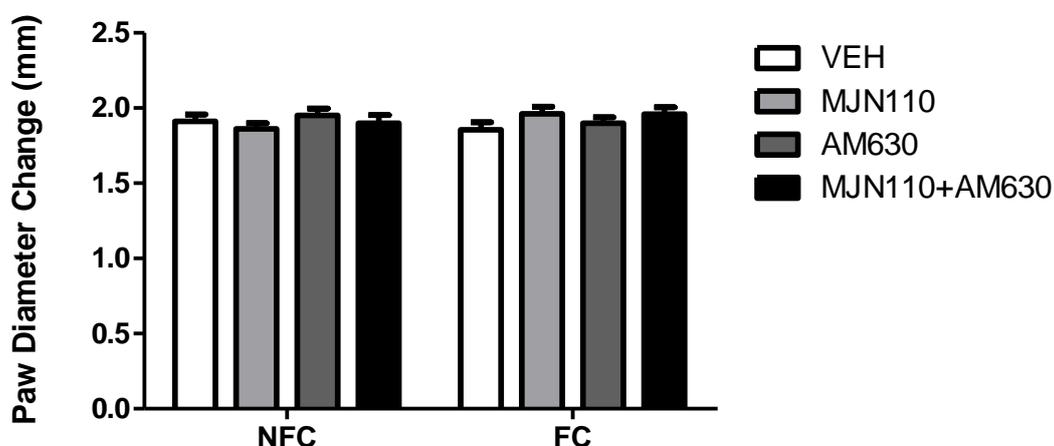


Figure 5.11: Effects of fear-conditioning and bilateral administration of MJN110, AM630 or MJN110+AM630 directly into the ACC on paw diameter change (mm) pre and post formalin injection (oedema). All data are expressed as mean \pm SEM ($n=8-10$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Two-way ANOVA revealed a significant effect of fear-conditioning ($F(1,63)=114.928$, $p<0.001$), treatment ($F(3,63)=35.188$, $p<0.001$) and fear-conditioning*treatment ($F(3,63)=19.022$, $p<0.001$) on CPS. Further post-hoc analysis revealed that re-exposure of rats to the arena previously paired with footshock resulted in a significant reduction in formalin-evoked nociceptive behaviour, confirming the expression of FCA (FC VEH vs. NFC VEH, $p<0.001$). Treatment with MJN110 attenuated the expression of FCA (FC VEH vs. FC MJN110, $p<0.001$), an effect blocked by co-administration with AM630 (FC MJN110 vs. FC MJN110+AM630, $p<0.001$). Treatment with AM630 alone reduced formalin-evoked nociceptive behaviour in NFC rats but had no effect in FC rats (NFC VEH vs. NFC AM630,

$p < 0.001$), an effect not observed upon co-administration with MJN110 (NFC VEH vs. NFC MJN110+AM630, $p = 0.853$; NFC AM630 vs. NFC MJN110+AM630). AM630 treated rats did not express FCA over the course of the 30-minute trial (FC AM630 vs. NFC AM630, $p = 0.963$) (Figure 5.12).

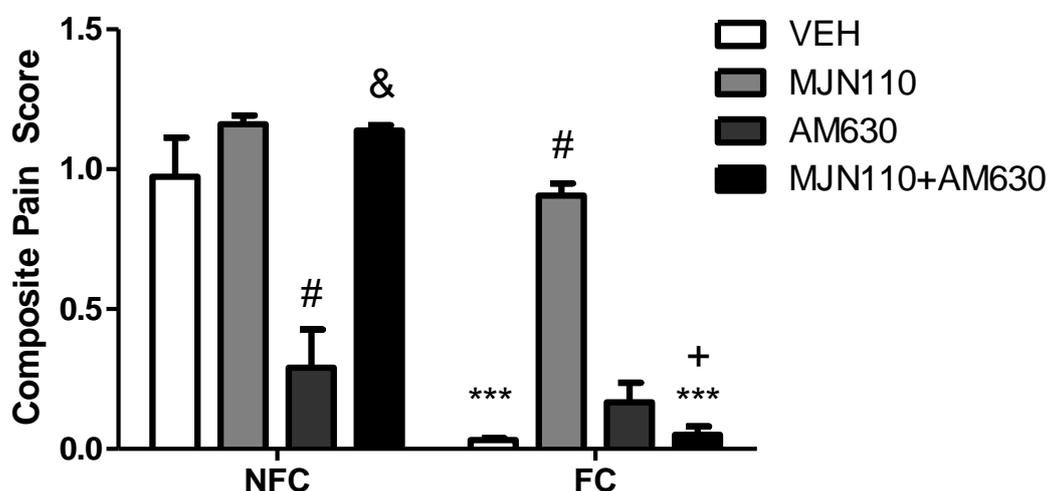


Figure 5.12: Effects of fear-conditioning and bilateral administration of MJN110, AM630 and MJN110+AM630 directly into the ACC on formalin-evoked nociceptive behaviour in rats over the full 30-minute testing period. *** $p < 0.001$ FC vs. NFC; # $p < 0.001$ vs. VEH counterpart; & vs. NFC AM630; + vs. FC MJN110 (Tukey's). All data are expressed as mean \pm SEM ($n = 8-10$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Temporal analysis of the data subdivided into 10-minute time bins and using 2-way repeated measures ANOVA revealed a significant main effect of fear-conditioning ($F(1,63) = 114.932$, $p < 0.001$), treatment ($F(3,63) = 35.189$, $p < 0.001$) and fear-conditioning*treatment ($F(3,63) = 19.022$, $p < 0.001$) on CPS. Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated ($\chi^2(2) = 27.283$, $p < 0.001$) and therefore a Greenhouse-Geisser correction was used. There was a significant main effect of time ($F(1.48, 92.92) = 6.890$, $p < 0.01$) and time*fear-conditioning ($F(1.48, 92.92) = 12.916$, $p = 0.000$), time*treatment ($F(6.0, 92.92) = 3.384$, $p < 0.01$) and time*fear-conditioning*treatment ($F(6.0, 92.92) = 3.084$, $p < 0.05$). Further post-hoc analysis revealed a significant reduction in formalin-evoked nociceptive behaviour in FC VEH treated rats for the entire 30-minute trial (FC VEH vs. NFC VEH, 0-10mins, 10-20mins, 20-30mins, $p < 0.001$) confirming the expression of FCA. Treatment with MJN110 attenuated the expression of FCA for the entire 30-minute testing

period (FC VEH vs. FC MJN110, 0-10mins, 10-20mins, 20-30mins, $p < 0.001$), an effect blocked by co-administration with AM630 (FC MJN110 vs. FC MJN110+AM630, 0-10mins, 10-20mins, 20-30mins, $p < 0.001$). Treatment with AM630 alone reduced formalin-evoked nociceptive behaviour in NFC rats for the entire 30-minute trial (NFC VEH vs NFC AM630, 0-10mins, 10-20mins, 20-30mins, $p < 0.001$), an effect not observed upon co-administration with MJN110 (NFC VEH vs. NFC MJN110+AM251, 0-10mins, 10-20mins, 20-30mins, $p = 1.000$; NFC AM630 vs. NFC MJN110+AM630, $p < 0.001$) (Figure 5.13).

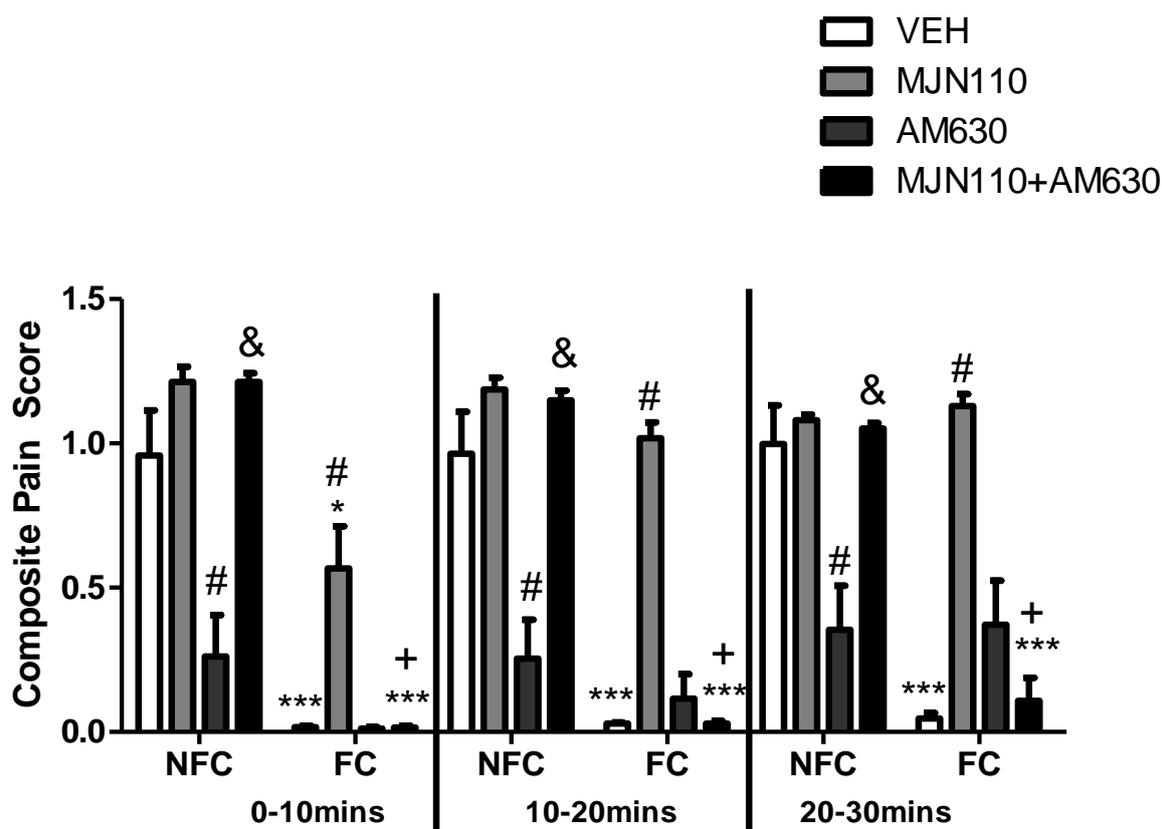


Figure 5.13: Effects of fear-conditioning and bilateral administration of MJN110, AM630 and MJN110+AM630 directly into the ACC on formalin-evoked nociceptive behaviour in rats over the full 30-minute testing period subdivided into 10-minute time bins. *** $p < 0.001$ FC vs. NFC; # $p < 0.001$ vs. VEH counterpart; & vs. NFC AM630; + vs. FC MJN110 (Tukey's). All data are expressed as mean \pm SEM ($n = 8-10$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

5.3.2.3 Effects of intra-ACC administration of MJN110, AM630 or MJN110+AM630 on expression of conditioned fear behaviour in the presence of formalin-evoked nociceptive tone

Duration of Freezing:

Kruskal Wallis comparisons revealed a significant main effect on the duration of freezing ($\chi^2(7)=56.334$, $p<0.001$) over the course of the 30-minute testing period. Further post-hoc analysis using Dunn-Bonferroni revealed that FC rats displayed significantly more freezing (FC VEH vs. NFC VEH, $p<0.001$) compared to NFC rats over the course of the 30-minute testing period. However, FC MJN110-treated rats did not display a significant increase in contextually induced freezing compared with NFC counterparts (FC MJN110 vs. NFC MJN110, $p=1.000$), (Figure 5.14). Intra-ACC MJN110 and AM630 alone or in combination had no significant effect on the duration of freezing.

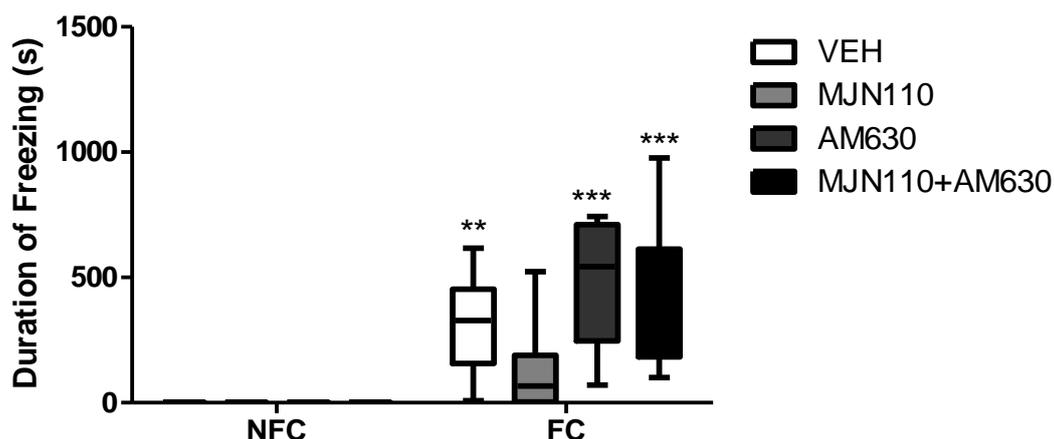


Figure 5.14: Effects of fear-conditioning and bilateral administration of MJN110, AM630 and MJN110+AM630 directly into the ACC on duration of freezing behaviour in rats over the full 30-minute testing period. ** $p<0.01$, *** $p<0.001$ FC vs. NFC (Dunn-Bonferroni). All data are expressed as median with interquartile range ($n=8-10$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Analysis of the data subdivided into 10-minute time bins using Kruskal-Wallis revealed a significant between-group effect on the expression of freezing at 0-10mins ($\chi^2(7)=56.122$, $p<0.001$), 10-20mins ($\chi^2(7)=31.075$, $p<0.001$) and 20-30mins ($\chi^2(7)=16.502$, $p<0.05$). Post-

hoc analysis using the Dunn-Bonferroni test revealed that fear-conditioned rats receiving intra-ACC VEH displayed significantly increased freezing behaviour in the first 10-minutes of the trial, compared with NFC VEH treated counterparts (FC VEH vs. NFC VEH; 0-10mins, $p < 0.01$). FC MJN110 treated rats did not display an increase in the duration of freezing (FC MJN110 vs. NFC MJN110, $p = 0.814$) despite the graphs showing a trend towards increase. Rats that received intra-ACC AM630 alone or in combination with MJN110 exhibited significant contextually induced freezing behaviour for a longer period of time (first 20 mins) than vehicle-treated fear-conditioned rats (first 10 mins only) (FC AM630 vs. NFC AM630, FC MJN110+AM630 vs NFC MJN110+AM630; 10-20mins, $p < 0.05$) (Figure 5.15).

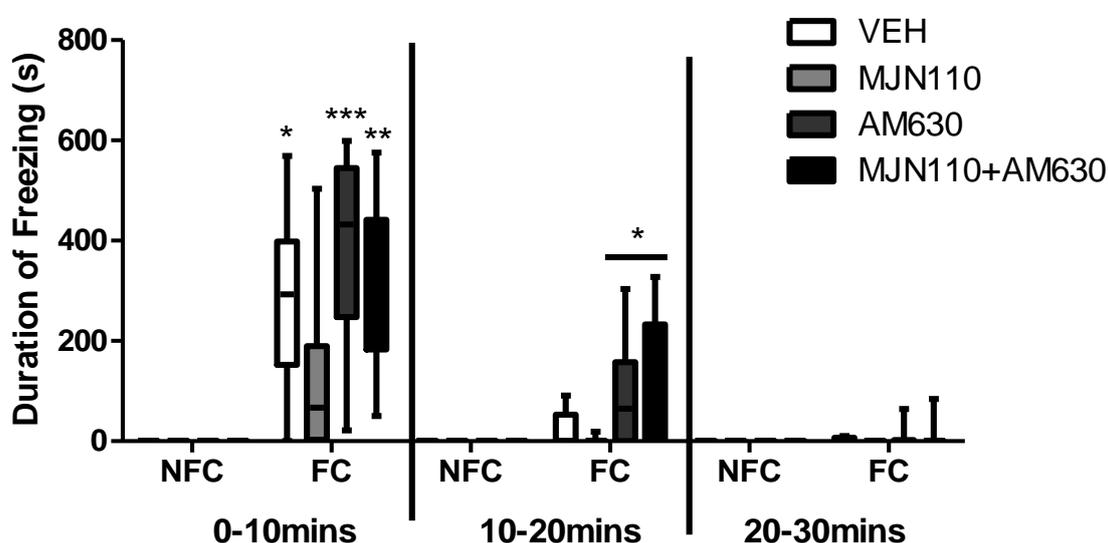


Figure 5.15: Effects of fear-conditioning and bilateral administration of MJN110, AM630 and MJN110+AM630 directly into the ACC on duration of freezing behaviour in rats over the full 30-minute testing period subdivided into 10-minute time bins. * $p < 0.05$, *** $p < 0.001$ FC vs. NFC (Dunn-Bonferroni). All data are expressed as median with interquartile range ($n = 8-10$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Ultrasonic Vocalisation:

Kruskal Wallis comparisons a significant main effect on the duration of 22kHz ultrasonic vocalization ($\chi^2(7) = 43.396$, $p < 0.001$) over the course of the 30-minute testing period. Further post-hoc analysis using Dunn-Bonferroni revealed that fear-conditioned rats expressed a

significantly higher duration of 22kHz ultrasonic vocalization (FC VEH vs. NFC VEH, $p < 0.05$) compared to their non-fear conditioned counterparts over the course of the 30-minute testing period. FC MJN110 treated rats did not display an increase in the duration of ultrasonic vocalisation (FC MJN110 vs. NFC MJN110, $p = 1.000$), despite the graphs showing a trend towards increase (Figure 5.16). Intra-ACC MJN110 and AM630 alone or in combination had no significant effect on the duration of 22kHz ultrasonic vocalisation.

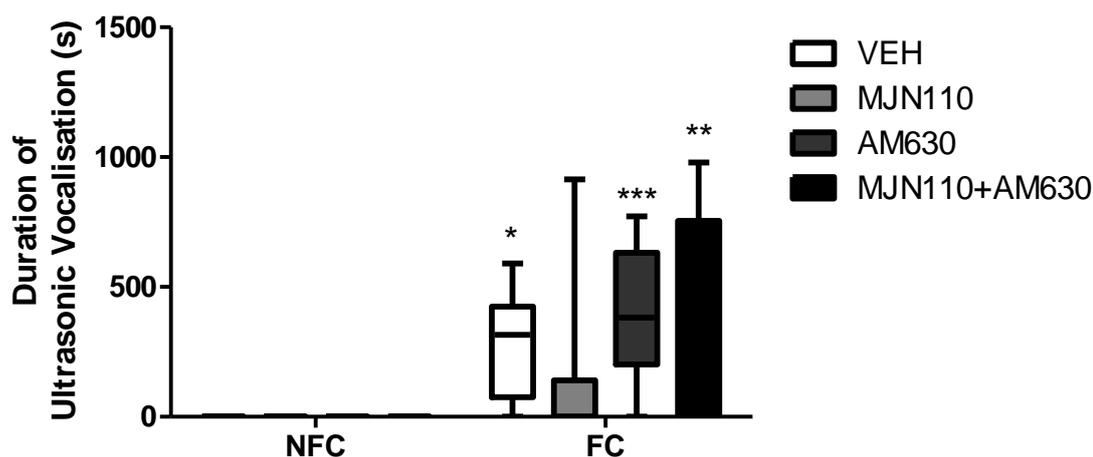


Figure 5.16: Effects of fear-conditioning and bilateral administration of MJN110, AM630 and MJN110+AM630 directly into the ACC on duration of ultrasonic vocalisation in rats over the full 30-minute testing period. ** $p < 0.01$, *** $p < 0.001$ FC vs. NFC (Dunn-Bonferroni). All data are expressed as median with interquartile range ($n = 8-10$) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Analysis of the data subdivided into 10-minute time bins using Kruskal-Wallis revealed a significant between-group effect on the expression of 22kHz ultrasonic vocalisation at 0-10mins ($\chi^2(7) = 45.647$, $p < 0.001$), 10-20mins ($\chi^2(7) = 21.298$, $p < 0.01$) but not 20-30mins ($\chi^2(7) = 11.769$, $p = 0.108$). Post-hoc analysis using the Dunn-Bonferroni test revealed that fear-conditioned rats receiving intra-ACC VEH displayed significantly increased 22kHz ultrasonic vocalisation behaviour in the first 10-minutes of the trial, compared with NFC VEH treated counterparts (FC VEH vs. NFC VEH; 0-10mins, $p < 0.01$). FC MJN110 treated rats did not display an increase in the duration of ultrasonic vocalisation (FC MJN110 vs. NFC MJN110, $p = 1.000$) despite the graphs showing a trend towards increase (Figure 5.17). Intra-ACC

MJN110 and AM630 alone or in combination had no significant effect on the duration of 22kHz ultrasonic vocalisation

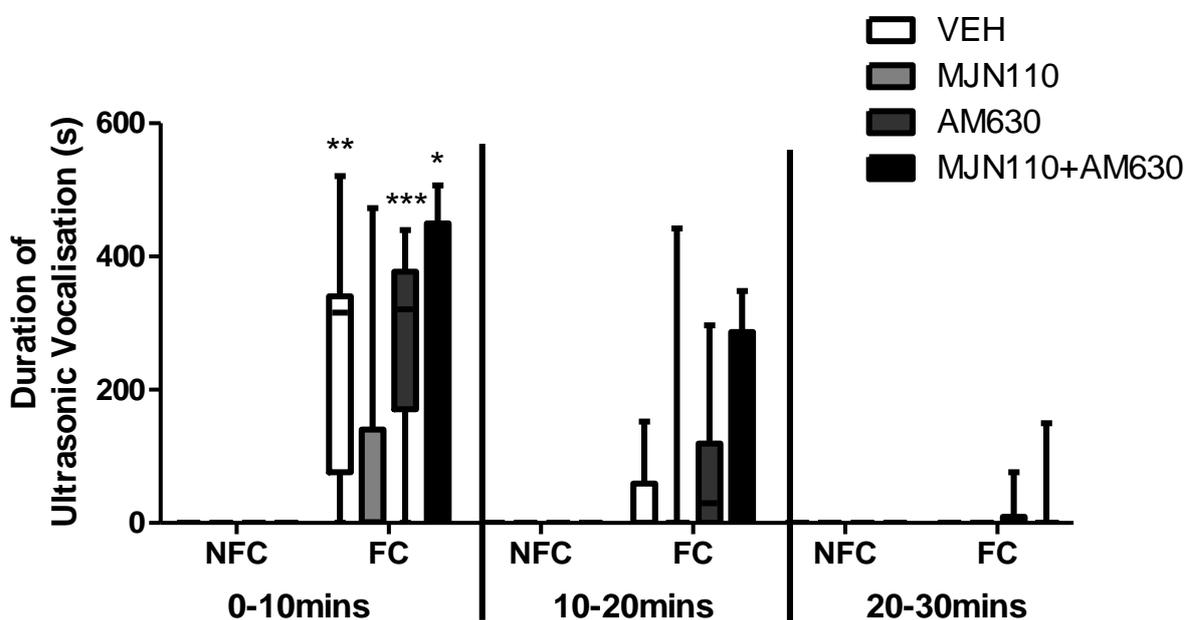


Figure 5.17: Effects of fear-conditioning and bilateral administration of MJN110, AM630 and MJN110+AM630 directly into the ACC on duration of the duration of ultrasonic vocalisation in rats over the full 30-minute testing period subdivided into 10-minute time bins. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ FC vs. NFC (Dunn-Bonferroni). All data are expressed as median with interquartile range ($n = 8-10$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

5.3.2.4 Effects of fear-conditioning and MJN110, AM630 or MJN110+AM630 treatment on locomotor behaviours and defecation in formalin-treated rats

Two-way ANOVA revealed a significant effect of fear-conditioning*treatment ($F(3,63) = 2.844$, $p < 0.05$) but not fear-conditioning ($F(1,63) = 1.570$, $p = 0.215$) or treatment ($F(3,63) = 0.928$, $p = 0.432$) on the distance moved over the 30-minute testing period. Further post-hoc analysis revealed no significant effects (Table 5.6).

Two-way ANOVA revealed a significant effect of treatment ($F(3,63) = 3.184$, $p < 0.05$) but not fear-conditioning ($F(1,63) = 1.404$, $p = 0.240$) or fear-conditioning*treatment ($F(3,63) = 1.101$, $p = 0.355$) on the duration of walking over the 30-minute testing period. Further post-hoc analysis revealed no significant effects (Table 5.6).

Two-way ANOVA revealed a significant effect of fear-conditioning ($F(1,63)=17.652$, $p<0.001$), treatment ($F(3,63)=7.087$, $p<0.001$) and fear-conditioning*treatment ($F(3,63)=9.864$, $p<0.001$) on the duration of rearing over the 30-minute testing period. Further post-hoc analysis revealed a significant increase in the duration of rearing over the course of the 30-minute testing period in FC VEH treated rats (FC VEH vs. NFC VEH, $p<0.001$) and effect attenuated by either MJN110 or AM630 alone but not in combination (FC MJN110 vs. NFC MJN110, $p=0.969$; FC AM630 vs. NFC AM630, $p=0.575$; FC MJN110+AM630 vs. NFC MJN110+AM630, $p<0.001$). FC AM630 or MJN110 alone also decreased the duration of rearing in FC rats compared to VEH treated rats, an effect blocked upon co-administration (FC VEH vs. FC MJN110, $p<0.001$; FC VEH vs. FC AM630, $p<0.01$; FC VEH vs. FC MJN110+AM630, $p=0.778$) (Table 5.6).

Two-way ANOVA revealed a significant effect of fear-conditioning ($F(1,63)=14.334$, $p=0.000$) and treatment ($F(3,63)=5.556$, $p<0.01$) but not fear-conditioning*treatment ($F(3,63)=1.817$, $p=0.153$) on the duration of grooming over the 30-minute testing period. Further post-hoc analysis revealed no significant effects (Table 5.6).

Kruskal Wallis comparisons revealed a significant effect ($\chi^2(7)=40.912$, $p<0.001$) for the amount of defecation in the arena (no. of pellets) over the 30-minute testing period. Further post-hoc analysis by Dunn-Bonferroni revealed that FC rats (FC-VEH vs. NFC VEH, $p<0.05$) exhibited a significant increase in defecation over the course of the 30-minute testing period compared to their NFC counterparts. Treatment with MJN110 and AM630 alone or in combination had no effect on the amount of defecation exhibited by rats (Table 5.6).

Treatment Group	Distance Moved (cm)	Walking (s)	Rearing (s)	Grooming (s)	Defecation (number of pellets)
NFC VEH	1968±96	91±13	31±17	66±27	3(3)
NFC MJN110	2187±297	83±24	2±1	30±10	3(2)
NFC AM630	2020±167	66±8	87±21	111±23	2(1)
NFC MJN110+AM630	1635±166	46±12	1±1	29±11	2(1)
FC VEH	2117±149	86±11	130±19***	135±36	6(2)*
FC MJN110	1581±132	54±21	25±12 #	46±14	6(4)*
FC AM630	1565±222	40±9	49±12#	158±29	6(2)*
FC MJN110+AM630	1906±140	60±8	98±16***	154±19	5(3)*

Table 5.6: Effects of fear-conditioning and MJN110 or MJN110+AM251 on distance moved, walking, rearing duration, grooming and defecation in formalin treated rats. * $p < 0.05$, *** $p < 0.001$ vs. NFC; # $p < 0.05$ vs FC VEH (Tukey's/Dunn-Bonferroni). All data are expressed as mean \pm SEM or median with interquartile range ($n=8-10$, per group) FC, fear-conditioned; NFC, non-fear-conditioned; VEH, vehicle.

5.3.3 Effect of fear-conditioning and vehicle or MJN110 on AEA, OEA, PEA or 2-AG levels in the mPFC of formalin-treated rats

5.3.3.1 Experiment 1 and 2:

Anterior Cingulate Cortex:

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,65)=0.230$, $p=0.663$), treatment ($F(1,65)=0.762$, $p=0.386$) or fear-conditioning*treatment ($F(1,65)=0.008$, $p=0.929$) on the levels of AEA in the ACC (Figure 5.18).

Two-way ANOVA revealed a significant effect of fear-conditioning ($F(1,71)=4136$, $p=0.046$) and treatment ($F(1,71)=4.451$, $p=0.038$) but not fear-conditioning*treatment ($F(1,71)=2.370$, $p=0.128$) on the levels of 2-AG in the ACC. Further post-hoc analysis revealed a significant

increase in the levels of 2-AG in MJN110-treated FC rats compared to FC VEH-treated rats (FC VEH vs. FC MJN110, $p < 0.05$). In contrast, MJN110 had no significant effect on levels of 2-AG in the ACC of NFC rats (Figure 5.18).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,68)=2.517$, $p=0.117$), treatment ($F(1,68)=0.403$, $p=0.528$) or fear-conditioning*treatment ($F(1,68)=0.054$, $p=0.817$) on the levels of PEA in the ACC (Figure 5.18).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,66)=1.397$, $p=0.241$), treatment ($F(1,66)=0.913$, $p=0.343$) or fear-conditioning*treatment ($F(1,66)=0.345$, $p=0.559$) on the levels of OEA in the ACC (Figure 5.18).

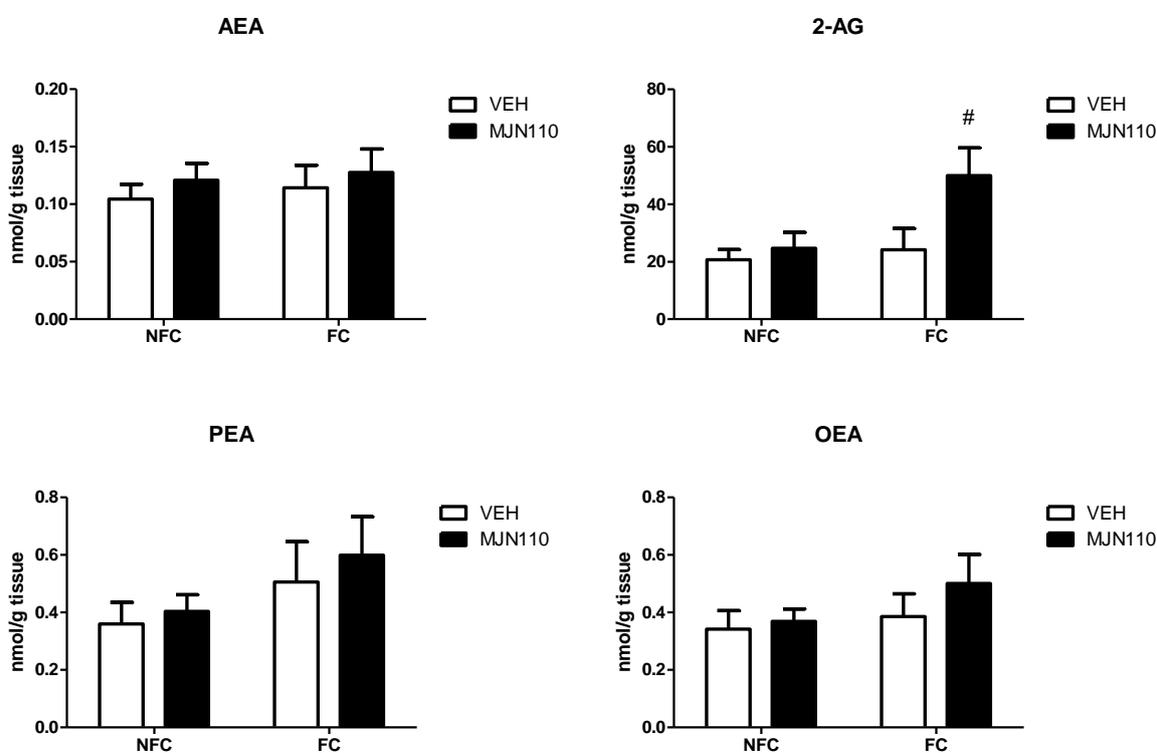


Figure 5.18: Effect of fear-conditioning and MJN110 on AEA, 2-AG, PEA or OEA levels in the ACC. # $p < 0.05$ vs FC VEH. All data are expressed as mean \pm SEM ($n=17-20$ per group); NFC-Non-fear-conditioned; FC-fear-conditioned; VEH-vehicle

Infralimbic Cortex:

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,70)=0.018$, $p=0.893$), treatment ($F(2,70)=0.332$, $p=0.566$) or fear-conditioning*treatment ($F(2,70)=0.690$, $p=0.409$) on the levels of AEA in the IL (Figure 5.19).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,72)=0.056$, $p=0.813$), treatment ($F(1,72)=0.210$, $p=0.648$) or fear-conditioning*treatment ($F(1,72)=0.042$, $p=0.838$) on the levels of 2-AG in the IL (Figure 5.19).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,71)=0.009$, $p=0.925$), treatment ($F(1,71)=0.091$, $p=0.763$) or fear-conditioning*treatment ($F(1,71)=0.357$, $p=0.552$) on the levels of PEA in the IL (Figure 5.19).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,71)=0.000$, $p=0.998$), treatment ($F(1,71)=0.241$, $p=0.625$) or fear-conditioning*treatment ($F(1,71)=0.330$, $p=0.568$) on the levels of OEA in the IL (Figure 5.19).

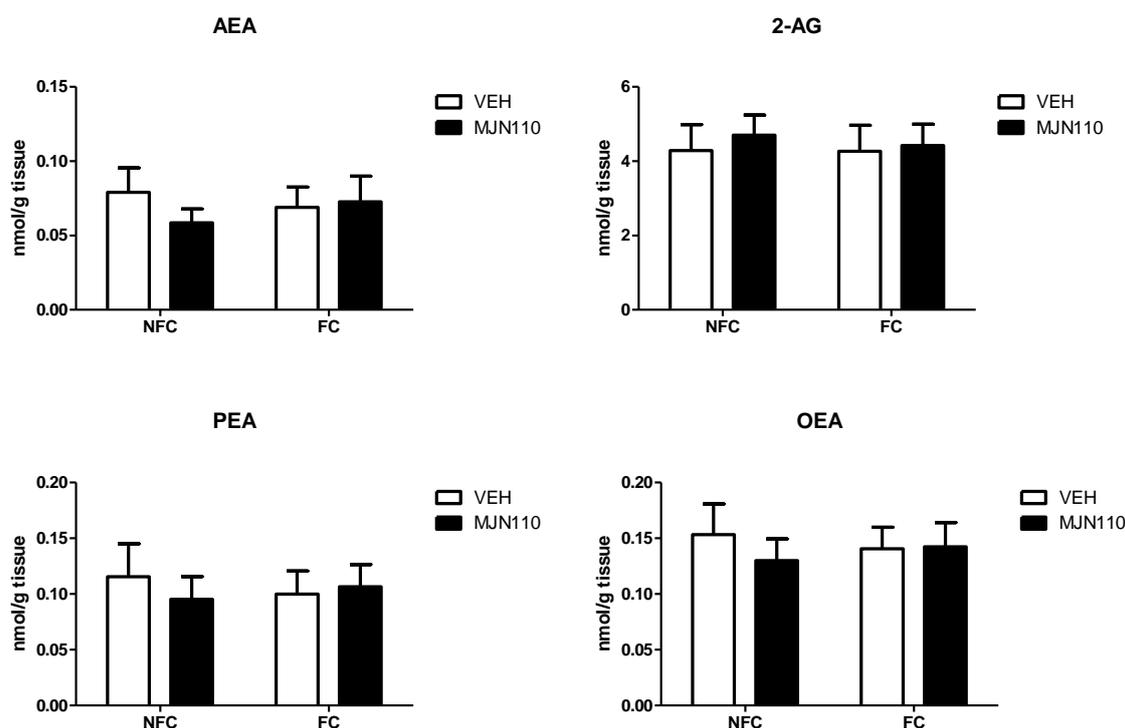


Figure 5.19: Effect of fear-conditioning and MJN110 on AEA, 2-AG, PEA or OEA levels in the IL. All data are expressed as mean \pm SEM ($n=17-20$ per group); NFC-Non-fear-conditioned; FC-fear-conditioned; VEH-vehicle

Prelimbic Cortex:

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,67)=0.409$, $p=0.525$), treatment ($F(1,67)=1.898$, $p=0.173$) or fear-conditioning*treatment ($F(1,67)=0.192$, $p=0.663$) on the levels of AEA in the PrL (Figure 5.20).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,72)=3.201$, $p=0.078$), treatment ($F(1,72)=0.163$, $p=0.688$) or fear-conditioning*treatment ($F(1,72)=0.139$, $p=0.710$) on the levels of 2-AG in the PrL (Figure 5.20).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,68)=3.018$, $p=0.087$), treatment ($F(1,68)=0.003$, $p=0.957$) or fear-conditioning*treatment ($F(1,68)=0.089$, $p=0.767$) on the levels of PEA in the PrL (Figure 5.20).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,71)=1.055$, $p=0.308$), treatment ($F(1,71)=0.278$, $p=0.600$) or fear-conditioning*treatment ($F(1,71)=1.942$, $p=0.168$) on the levels of OEA in the PrL (Figure 5.20).

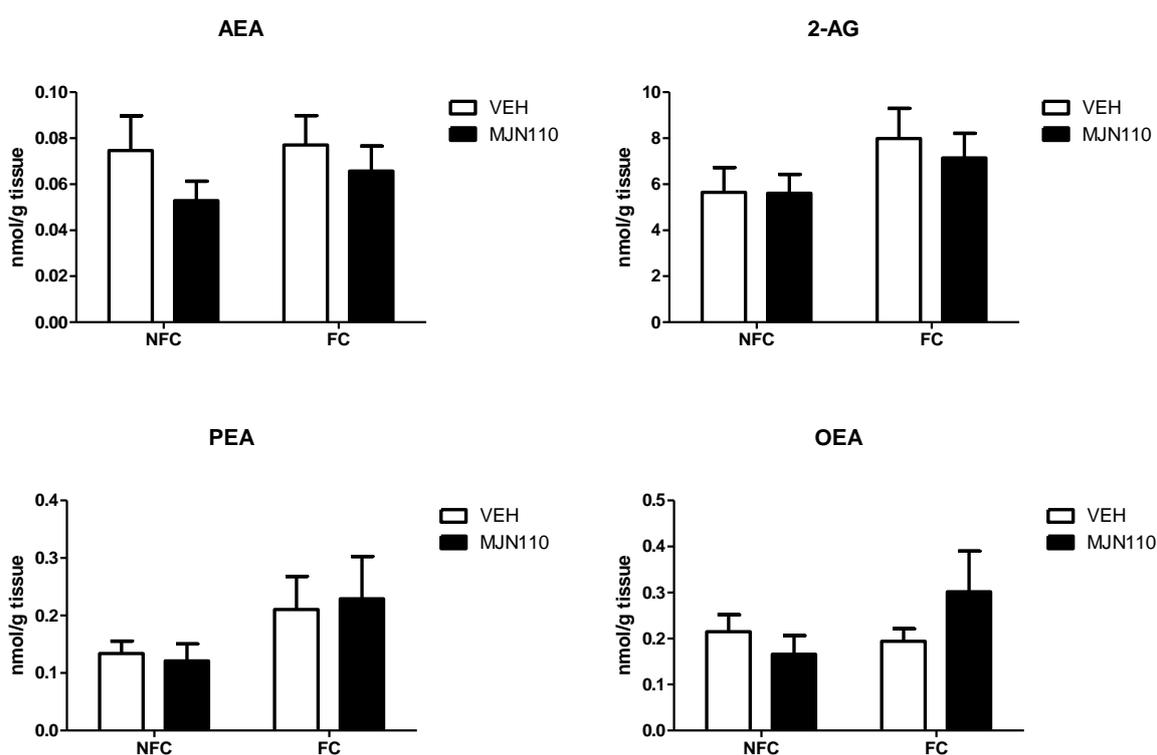


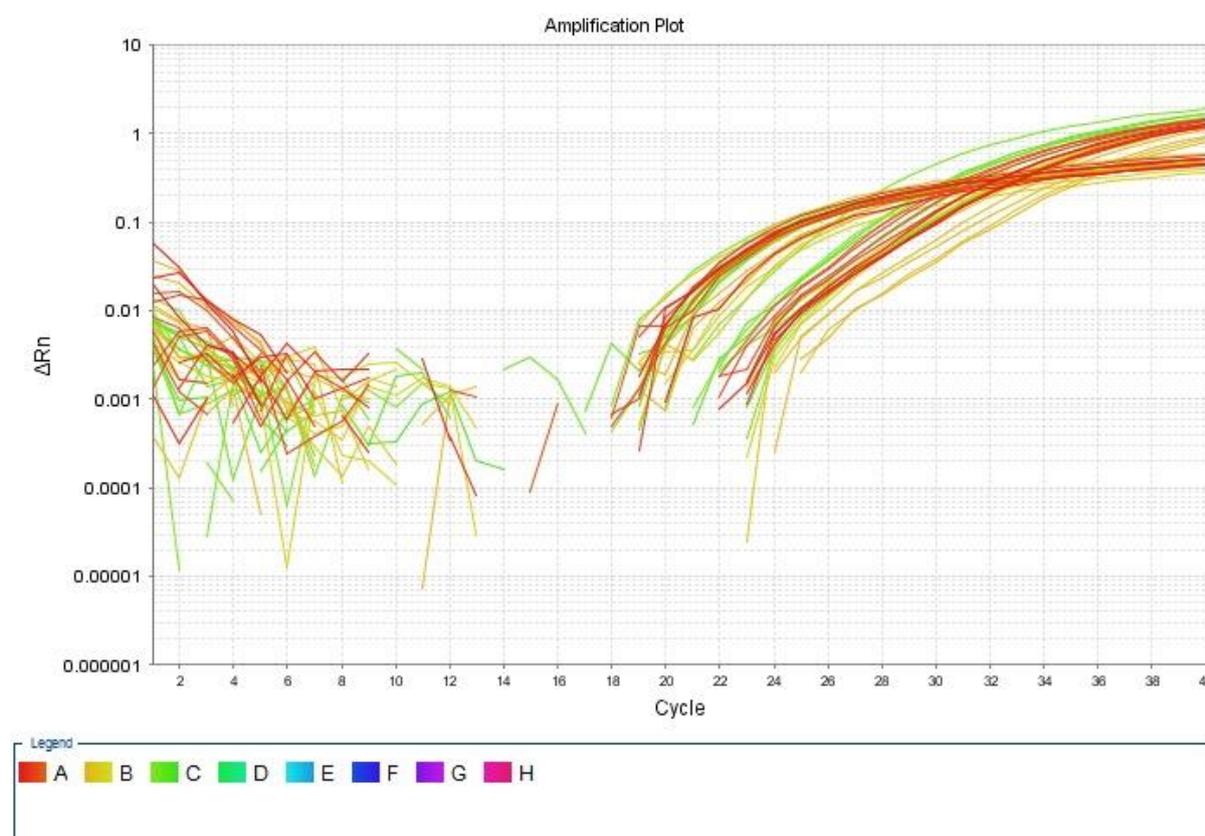
Figure 5.20: Effect of fear-conditioning and MJN110 on AEA, 2-AG, PEA or OEA levels in the PrL. All data are expressed as mean \pm SEM (n=15-20 per group); NFC-Non-fear-conditioned; FC-fear-conditioned; VEH-vehicle

5.3.4 Determination of the presence of CB₂ in the infralimbic, prelimbic and anterior cingulate cortices of the medial prefrontal cortex

In order to determine the presence of CB₂ in the three subregions of the mPFC, quantitative real-time PCR was carried out using a FAM-labelled CB₂ primer and a VIC-labelled GAPDH housekeeping primer. From the amplification plot (Figure 5.21) and CT values for CB₂ mRNA (Table 5.7) CB₂ transcript is present in these mPFC subregions, but at low amounts.

Region	Average CT values for CB ₂
IL	31
PrL	32
ACC	30

Table 5.7: CT values for CB₂ mRNA expression in the mPFC. IL: infralimbic cortex; PrL: prelimbic cortex; ACC: anterior cingulate cortex. All data are expressed as mean \pm SEM (n=6-9 per group)



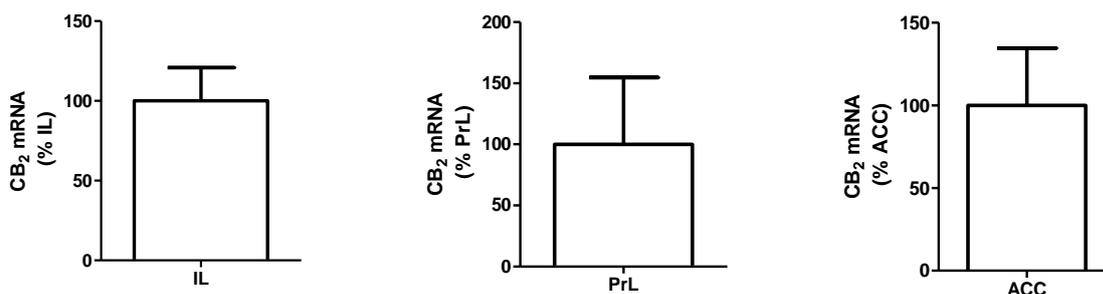


Figure 5.21: Amplification plot and graphical representation of CB₂ mRNA expression in the mPFC. IL: infralimbic cortex; PrL: prelimbic cortex; ACC: anterior cingulate cortex. All data are expressed as mean \pm SEM (n=6-9 per group)

5.3.5 Effect of fear-conditioning on the levels of CB₁ and CB₂ mRNA expression in the ACC of rats

The results from the t-test revealed that fear-conditioning had no effect on levels of CB₁ ($t(14)=-0.253$, $p=0.804$) or CB₂ ($t(14)=0.908$, $p=0.379$) mRNA in the ACC, measured at the end of the 30-minute test trial (Figure 5.22).

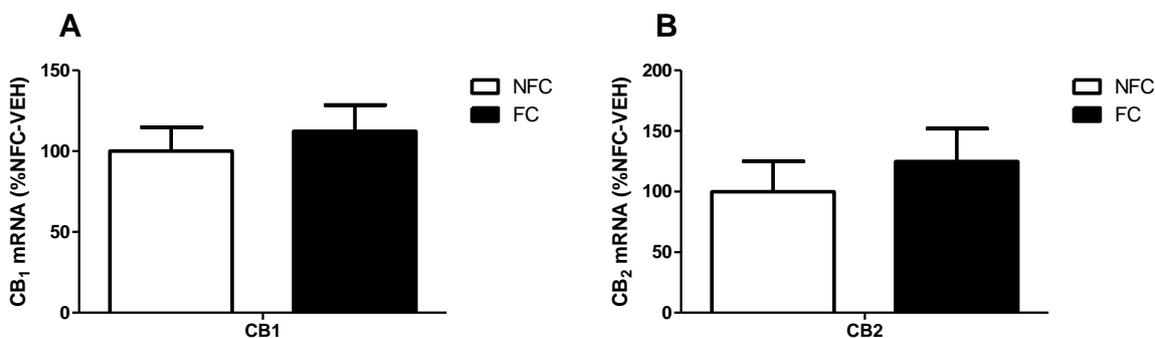


Figure 5.22: Levels of (A) CB₁ and (B) CB₂ mRNA in the ACC of FC and NFC VEH treated rats. FC: fear-conditioned; NFC: Non-fear-conditioned.

5.4 Discussion

The data presented herein indicate for the first time that the MAGL substrate 2-AG in the rat ACC modulates fear-pain interactions via a possible CB₂ receptor-mediated mechanism. In the absence of contextual fear conditioning, administration of the MAGL inhibitor alone or in combination with the CB₁ receptor antagonist AM251 or CB₂ receptor antagonist AM630 had no effect on formalin-evoked nociceptive behaviour. In the absence of contextual fear conditioning, intra-ACC AM630 decreased formalin-evoked nociceptive behaviour, an effect blocked upon co-administration with MJN110. Re-exposure of vehicle-treated rats to a context previously paired with foot shock significantly reduced formalin-evoked nociceptive behaviour (i.e. induced FCA). Microinjection of the MAGL inhibitor MJN110 into the ACC attenuated the expression of FCA, an effect unopposed upon co-administration with AM251 but blocked by co-administration with AM630. Re-exposure to a context previously paired with an aversive stimulus increased the expression of freezing and 22kHz ultrasonic vocalisation in the presence of nociceptive tone. Rats that received intra-ACC AM630 alone or in combination with MJN110 exhibited significant contextually induced freezing behaviour for a longer period of time than vehicle-treated fear-conditioned rats. Mass spectrometry revealed that intra-ACC MJN110 increased the levels of 2-AG in the ACC of FC rats compared to FC VEH treated rats. Neither FC nor intra-ACC administration of MJN110 were associated with alterations of endocannabinoids or related n-acylethanolamines in the PrL or IL at the end of the 30-minute trial. These data demonstrate for the first time that the ACC is an important neural substrate regulating the expression of FCA and fear-pain interactions by a possible 2-AG-CB₂ mediated mechanism.

Regardless of experiment, VEH-treated FC rats exhibited a reduction in formalin-evoked nociceptive behaviour upon re-exposure to a context previously paired with an aversive stimulus (i.e. footshock), confirming the expression of FCA. This finding is consistent with the three earlier chapters and a number of previous studies, within our own group and others, investigating FCA (Fanselow, 1984, Finn et al., 2004, Olango et al., 2012, Rea et al., 2013, Roche et al., 2007, Helmstetter, 1992, Helmstetter and Bellgowan, 1993, Rea et al., 2014a, Rea et al., 2009).

In both experiments described in this chapter, MAGL inhibition via MJN110 attenuated FCA while increasing the expression of formalin-evoked nociceptive behaviour in FC rats. This effect was not blocked by the CB₁ receptor antagonist AM251 but was blocked by the CB₂ receptor antagonist AM630. These findings together suggest that FCA is attenuated by 2-AG

acting on CB₂ receptors in the ACC and are the first to suggest that suggest that the ACC is an important neural substrate regulating the expression of FCA alongside the ventral hippocampus (Ford et al., 2011), dIPAG (Olango et al., 2012), BLA (Rea et al., 2013, Roche et al., 2007), IL and PrL (Rea et al., 2018).

In experiment 1, the effects of MJN110 were not blocked by co-administration of AM251. These results suggest that 2-AG modulates the expression of FCA in the ACC via a non-CB₁ receptor target. As discussed in chapters 3 and 4, other non-CB₁ targets (Baggelaar et al., 2018) could include CB₂ (Gonsiorek et al., 2000, Sugiura et al., 2000), GABA_A (Sigel et al., 2011), PPARs (Bouaboula et al., 2005), adenosine A₃ (Lane et al., 2010), TRPV1 (Yusaku et al., 2008) and GPR55 (Ryberg et al., 2007). To test the hypothesis that 2-AG modulates FCA by acting on CB₂ receptors, we reproduced this experiment with the CB₂ antagonist AM630. AM630 blocked the attenuation of FCA when co-administered with MJN110. This result suggests that 2-AG acts via CB₂ to attenuate FCA and is the first to provide evidence for a role of CB₂ in the ACC in FCA.

Chapter 2 investigated the role of FAAH substrates in the ACC in FCA. FAAH inhibition via URB597 and CB₁ receptor blockade via AM251 alone or in combination had no effect on the expression of FCA in the ACC. In contrast, the results of the present chapter suggest an important role for the endocannabinoid 2-AG, in the ACC in FCA. These results also indicate differential roles for FAAH substrates and 2-AG in the ACC in FCA, whereby FAAH substrates appear not to modulate the expression of FCA while 2-AG is involved in the attenuation of FCA. Moreover, both studies suggest that in the ACC, CB₂ and not CB₁ is involved in the expression of FCA. Neither CB₂ nor CB₁ receptor antagonism had any effect on FCA alone, however CB₂ receptor antagonism via AM630 blocked the effect of the MAGL inhibitor MJN110.

Rea et al., and chapter 2 of this thesis found that the FAAH inhibition via URB597 had differential effects in the subregions of the mPFC (IL, PrL and ACC) (Rea et al., 2018). In contrast, the results of this chapter are comparable with those observed in chapters 3 and 4. Regardless of the subregion, microinjection of the MAGL inhibitor MJN110 into the mPFC functions to attenuate FCA. These results suggest that while FAAH substrates may have differential effects depending on the subregion of the mPFC it is administered to, the function of 2-AG remains consistent. Future studies investigating and comparing the mechanisms and

receptors by which these two drugs exert their effects in the mPFC are necessary to fully elucidate their roles in FCA.

In experiment 2, intra-ACC administration of AM630 decreased formalin-evoked nociceptive behaviour in NFC rats, an effect blocked upon co-administration with MJN110. This finding provides evidence that CB₂ receptors in the ACC may facilitate the expression of formalin-evoked nociceptive behaviour in rats, an effect dependent on the levels of 2-AG present. In contrast to the result presented in this experiment, peripheral and spinal activation of CB₂ receptors has been shown to be antinociceptive in various models of pain including neuropathic, thermal and inflammatory (Ibrahim et al., 2003, Malan et al., 2001, Nackley et al., 2003, Quartilho et al., 2003, Clayton et al., 2002) in mice and rats respectively. However, few studies have investigated the role of CB₂ receptors supraspinally. Jhaveri et al., found that the CB₂ agonist JWH-133 injected directly into the central posterior nucleus of the thalamus reduced both non-noxious and noxious mechanically evoked responses of thalamic neurons in a rat model of neuropathic pain, an effect blocked by CB₂ antagonism via SR144528. Alone CB₂ antagonism increased the burst activity of noxious but not non-noxious neuronal activity in the thalamus of spinal nerve ligated rats (Jhaveri et al., 2008a). The difference between the results presented in this chapter and those presented above may be due to region related effects and CB₂ receptor localisation. It is possible that CB₂ receptors in the ACC play a pro-nociceptive role, facilitating formalin-evoked nociceptive behaviour. The precise expression and subcellular localisation of CB₂ in the brain and subregions of the mPFC is still under investigation. Whether CB₂ expression is presynaptic or postsynaptic remains to be determined. den Boon et al., state that CB₂ is located intracellularly in layer II/III of pyramidal cells of the rodent mPFC, acting to inhibit neuronal excitability (den Boon et al., 2014, den Boon et al., 2012). It is possible that CB₂ acts postsynaptically in the mPFC to reduce neuronal excitability and facilitate formalin-evoked nociceptive behaviour. Pyramidal cell output has been shown to be decreased in the mPFC of a rat model of arthritis, leading to cognitive impairments such as decision making (Ji and Neugebauer, 2011, Ji et al., 2010). In this model, BLA hyperactivity deactivates the mPFC through glutamate-driven synaptic inhibition. Increasing pyramidal cell output via mGluR5 or CB₁ receptor activation in the mPFC reduces cognitive deficits, an effect potentiated if 2-AG-CB₁ signalling in the mPFC is restored (Kiritoshi et al., 2016, Kiritoshi et al., 2013). It is possible that CB₂ in the ACC decreases neuronal excitability, facilitating formalin-evoked nociceptive behaviour. This effect is no longer seen when 2-AG endogenous tone is increased via co-administration with the MAGL

inhibitor MJN110. 2-AG may act on CB₁ to inhibit GABAergic neurotransmission and increase pyramidal cell output thus attenuating the effect of CB₂. No studies to date have looked at CB₂ receptor expression on presynaptic GABAergic neurons in the mPFC. It is possible that CB₂ is also acting on these neurons to decrease inhibitory output and thus increase excitability from the mPFC leading to the facilitation of formalin-evoked nociceptive behaviour. As neural activity is increased in the ACC of humans, primates, rodents and rabbits in response to a noxious stimulus (Sikes and Vogt, 1992, Hutchison et al., 1999, Koyama et al., 1998, Yamamura et al., 1996, Baliki et al., 2006a) CB₂ acting on GABAergic interneurons in the ACC could contribute to this. Further studies aimed at elucidating the precise neurochemical and molecular mechanisms underpinning pain regulation in the absence of fear by CB₂ receptors in the ACC and other supraspinal regions are warranted.

In both experiments re-exposure to a context previously paired with footshock resulted in robust freezing behaviour and 22kHz ultrasonic vocalisation. In experiment 1, intra-ACC MJN110 or MJN110+AM251 had no effect on the expression of fear-related behaviours. This corroborates the work presented in chapters 3 and 4, where these drugs had no effect in the IL and PrL on fear-related behaviours in the presence of nociceptive tone. This is comparable to our previous studies (Finn et al., 2004, Rea et al., 2013, Roche et al., 2010, Roche et al., 2007) and those of others (Helmstetter and Fanselow, 1987, Kinscheck et al., 1984) indicating that FCA can be altered independently of the expression of freezing in these animals.

AM630 had no effect on the expression of FCA but rats administered intra-ACC AM630 expressed significant contextually induced freezing behaviour for a longer period of time than FC VEH treated rats, an effect unopposed by co-administration with MJN110, indicating a role for CB₂ receptors independent of 2-AG levels in the ACC in the termination of fear-related behaviours. It is possible that CB₂ in the ACC is involved in short-term within-trial extinction of fear, however to date there are a paucity of studies investigating the role of CB₂ receptors in fear extinction (Ruehle et al., 2012) and systemic administration of AM630 had no effect on freezing behaviour in mice exposed to cued fear conditioning procedure (Li and Kim, 2016). The prolongation of fear seen following CB₂ antagonism could imply a potential anxiolytic effect of CB₂ receptor signalling in the ACC. Systemic administration of the CB₂ receptor agonist β -caryophyllene produced anxiolytic effects in the open field, elevated plus and marble burying test in mice (Bahi et al., 2014). Similarly, mutant *Fmr1*-knockout mice exhibit behavioural fragile X phenotype which consists of an anxiolytic phenotype. Systemic AM630 attenuated the anxiolytic phenotype of these mice, indicated a role for CB₂ receptors in the

expression of this phenotype (Busquets-Garcia et al., 2013). Moreover, systemic MAGL inhibition via JZL184 produced anxiolytic effects in the elevated zero and plus maze tests, an effect abolished by pre-treatment with either SR144528 or AM630, indicated an anxiolytic effect of 2-AG at CB₂ receptors (Busquets-Garcia et al., 2011). This contrasts the results presented in this chapter which found the effect of CB₂ receptor blockade to be independent of 2-AG levels. Similarly, increasing the levels of 2-AG via MAGL inhibition alone did not produce an anxiolytic effect in the experiments presented here, although there were trends for a decrease in fear-related behaviours in FC rats in experiment 2 following MJN110 treatment. It should be noted that the studies described above looked at anxiety-related behaviours in the absence of pain while the experiments described in this chapter looked at fear-related behaviours in the presence of pain and thus a direct comparison cannot be made. Therefore, further studies are warranted to fully elucidate the role of CB₂ and 2-AG in the ACC during fear-conditioning in the absence of pain.

As seen in the IL and PrL, intra-ACC administration of MJN110 in experiment 1 decreased the distance moved in FC rats compared to VEH but had no effect on the duration of walking. This effect was not blocked by co-administration with AM251, indicating a role for a non-CB₁ receptor target. There was no drug or FC effects on distance moved in experiment 2, although significant trends existed for MJN110 and AM630 treated FC rats, suggesting an experimental difference in rats. Although the testing parameters (arena, holding room, temperature, food etc) were the same for both experiments this discrepancy may be due to noise related issues in the testing unit due to numerous experiments running simultaneously throughout the laboratory during experiment 2. Moreover, in experiment 1 fear-conditioning increased the duration of grooming in VEH treated rats, an effect blocked by intra-ACC MJN110 or MJN110+AM251, indicating an anxiolytic effect of this drug at a non-CB₁ receptor target. This is also comparable to the effects on grooming following MAGL inhibition in the PrL. In experiments 1 and 2, fear-conditioning increased the duration of rearing in VEH treated rats, an effect attenuated following MJN110 or AM630 treatment. Co-administration of MJN110 and AM251 did not reverse the MJN110 effect on rearing but co-administration of MJN110 and AM630 did, indicating the anxiolytic role for MJN110 on rearing behaviour in the ACC is via CB₂ receptors.

In an attempt to determine the mechanism underlying the effects of MAGL inhibition in the ACC on FCA we measured the levels of the endocannabinoids AEA and 2-AG and related N-acylethanolamines OEA and PEA locally in the ACC, IL and PrL. Samples from both

experiments were combined for analysis by mass spectrometry. The ACC is a large region spanning the length of Bregma 3.70mm to Bregma -0.26mm. In order to accurately determine the effect of MAGL inhibition on the tissue levels in this region punches were taken from ACC tissue where dye was present, approximating the spread of drug. Intra-ACC administration of MJN110 did not alter the levels of endocannabinoids or related N-acylethanolamines in the PrL or IL. This is comparable to the previous 2 chapters where MJN110 had no effect on the levels of endocannabinoids or related N-acylethanolamines in the regions of the mPFC where the drug was not injected. This implies that the effects of the drug are localised to the region to which it is injected. In contrast to the IL and PrL (chapters 3 and 4), intra-ACC administration of MJN110 increased the levels of 2-AG in FC rats compared to VEH. This is comparable to Sticht *et al.*, who found a significant increase in 2-AG levels following MJN110 administration into the visceral insular cortex (Sticht *et al.*, 2016) and implies that the effects of MJN110 in fear-conditioned rats are related to the increased levels of 2-AG in the ACC. Moreover, MJN110 had no effect on 2-AG levels in NFC rats. This suggests that the increases in 2-AG are only upon exposure to a fearful stimulus. Studies have shown that the levels of 2-AG are increased in rats exposed to fearful/stressful stimuli (Bluett *et al.*, 2017, Morena *et al.*, 2015, Patel *et al.*, 2005). This may be why we only see an increase in 2-AG in FC rats. It is possible that 2-AG has a protective function upon exposure to fearful and stressful stimuli and increased levels are an endogenous coping mechanism to decrease fear.

The expression of CB₂ receptors throughout the brain has been much less well established and characterized in comparison to CB₁ receptors. Initially it was thought that CB₂ is a peripherally restricted receptor. However, recent studies have demonstrated that CB₂ receptors are expressed in numerous brain regions including the olfactory cortex, cerebral cortex, striatum, thalamic nuclei, inferior colliculus, hippocampus, amygdala and PAG (Gong *et al.*, 2006, Van Sickle *et al.*, 2005). While CB₂ receptor expression has been demonstrated in the PFC and mPFC (Choi *et al.*, 2012, den Boon *et al.*, 2012), the study described in the present chapter used quantitative real-time PCR to determine if CB₂ mRNA could be detected within the three subregions of the mPFC, (IL, PrL and ACC). The results indicate a significant amplification of CB₂ mRNA within each of the mPFC regions although the high CT values imply that the levels within each of these regions are low. Moreover, there is no effect of fear-conditioning on the expression of either CB₁ or CB₂ in the ACC of rats. Our behavioural data indicate that while CB₁ in the ACC was not responsible for the effects of MJN110 on FCA, CB₂ receptors were, as the effects of MJN110 were blocked following CB₂ antagonism. Similarly, in chapters 3 and

4, the effects of MJN110 in the IL and PrL were not affected following coadministration with a CB₁ antagonist AM251, indicating a role for another cannabinoid related receptor. The results from the present study along with the evidence for CB₂ mRNA in the IL and PrL suggest that the effects of MJN110 in the IL and PrL may also be mediated via CB₂ receptors. However, as 2-AG was not increased in these regions, as it was in the ACC, following MJN110 treatment the effects in those studies may be due to a receptor other than CB₂. Future studies should look at the role of these receptors in the IL and PrL in the expression of FCA. Furthermore, further analysis of IL, PrL and ACC tissue using western blotting techniques are necessary to determine the CB₂ protein levels in these regions in the IL, PrL and ACC. For western blotting, the key limitation is the sensitivity and specificity of the antibody used. Current antibodies for CB₂ lack specificity and problems may arise from the difficulty of producing reliable antibodies against CB₂ (Atwood and Mackie, 2010, Dhopeswarkar and Mackie, 2014, Marchalant et al., 2014).

In conclusion, the present study provides new evidence to support a role for the endocannabinoid system in the ACC, in the expression of FCA, and conditioned fear in the presence of nociceptive tone. The data indicate that FCA is attenuated by 2-AG-CB₂ receptor signalling in the ACC, and that CB₂ receptors in the ACC may facilitate formalin-evoked nociceptive behaviour and terminate fear-related behaviours. Our data also suggest that the role of a MAGL substrate (likely 2-AG) in different subregions of the mPFC in FCA is conserved. This contrasts with the role of FAAH substrates in FCA in the three subregions of the mPFC. Therefore, elucidation of the role of the endocannabinoid system in these and other supraspinal brain regions in pain-fear interactions may facilitate increased understand and development of new therapeutic approaches for pain- and fear-related disorders and their comorbidity.

Chapter 6 : Reduced expression of fear-conditioned analgesia and conditioned fear-related behaviours in the stress and pain hyper-responsive Wistar-Kyoto rat strain and associated alterations in the endogenous cannabinoid system in the mPFC

6.1 Introduction

Pain can be defined as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage” (International Association for the Study of Pain [IASP] Task Force on Taxonomy, 1994). Recent data indicate that approximately 20% of the population suffer from chronic pain, the majority of whom also suffer from some other disability or mood disturbance (Blyth et al., 2001, Demyttenaere et al., 2007, Vos et al., 2012). Chronic pain is usually defined as pain persisting for over 3 months. It may be neuropathic, inflammatory, or idiopathic in nature (Aguggia, 2003). Epidemiological studies of 289 diseases and injuries concluded that chronic pain conditions were among the 10 conditions resulting in the longest number of years lived with disability (Vos et al., 2012). Patients with chronic pain often exhibit comorbid depression or anxiety disorders (McWilliams et al., 2003). Depression and anxiety can influence the way in which people experience pain, often enhancing the physical symptoms of pain (Raftery et al., 2011). There has recently been a growing interest in the interaction between pain and stress/anxiety. Stress can also exert an important influence on pain in one of two ways with intense, acute stress reducing perceived pain, stress-induced analgesia, and prolonged, repeated stress often increasing perceived pain, stress-induced hyperalgesia (Butler and Finn, 2009, Jennings et al., 2014).

The WKY rat is a useful model to study the influence of genetic background on co-morbid pain and stress disorders. The inbred WKY rat is a genetic variation of the Wistar rat strain (Okamoto and Aoki, 1963). It is a stress and pain hyper-responsive rat model that exhibits a co-morbid depressive and anxiogenic phenotype (Burke et al., 2010). In comparison to commonly used control comparators (such as the SD rat strain) WKY rats display increased depressive and anxiety-like behaviour in the forced swim test, open field test and elevated plus maze test (Burke et al., 2010, Gentsch et al., 1987, Pare, 1992). WKY rats also exhibit enhanced stress hormone and HPA axis responses to stress when compared to control comparator rat strains (Rittenhouse et al., 2002, Redei et al., 1994, De La Garza and Mahoney, 2004). In

addition, WKY rats display enhanced mechanical allodynia (Taylor et al., 2001, Robbins et al., 2007), nociceptive responding to visceral stimulation (Gunter et al., 2000, Gibney et al., 2010), exacerbated mechanical allodynia following peripheral nerve injury (Zeng et al., 2008) and enhanced inflammatory pain-related behaviour following intra-plantar injection of formalin (Burke et al., 2010, Rea et al., 2014b, Jennings et al., 2015, Madasu et al., 2016). Overall, there is a weight of evidence that suggest WKY rats display a stress and pain hyper-responsive profile. Thus, the WKY strain is a useful genetic model to study co-morbid pain and stress-related disorders.

The mPFC is comprised of three functionally distinct subregions, the IL, PrL and ACC. All three of these regions and their role in pain and fear have been discussed in detail in the previous 4 chapters. Components of the endocannabinoid system are widely expressed in the mPFC (Herkenham, 1991, Tsou et al., 1998a). The endocannabinoid system plays a key role in the modulation of nociception (Corcoran et al., 2015, Woodhams et al., 2017). FCA is a paradigm that allows for the study of endocannabinoid-mediated endogenous analgesia. Systemic administration of the FAAH inhibitor URB597 enhances the expression of FCA in rats while systemic administration of the CB₁ receptor antagonist/inverse agonist rimonabant or AM251 attenuates FCA (Finn et al., 2004, Olango et al., 2014, Butler et al., 2008). Endocannabinoid-mediated endogenous analgesia may be reduced in WKY vs. SD rats. In particular, the RVM and PAG represent key neural substrates where cannabinoid-mediated analgesia is impaired in WKY rats. Rea et al., demonstrated that the endocannabinoid system in the RVM is dysfunctional in WKY rats thereby contributing to their hyperalgesic phenotype following formalin-induced pain (Rea et al., 2014b). Similarly, alterations in the endocannabinoid system within the PAG of the WKY rat model may contribute to their hyperalgesic phenotype (Jennings, 2015a). To date, FCA has not been investigated in WKY rats. In addition, the role of the endocannabinoid system in the mPFC in pain and FCA in WKY rats has not yet been investigated.

Overall, this chapter examines the hypothesis that WKY rats have an impairment in their descending inhibitory pain pathway that will be evidenced by an impaired expression of FCA and increased expression of formalin-evoked nociceptive behaviour. The endocannabinoid system in the mPFC may contribute to the hyperalgesic phenotype of the WKY rats.

The overall aims of the study described in this chapter were:

- To determine whether there are strain differences in the expression of fear, formalin-evoked nociceptive behaviour and FCA in SD and WKY rats.
- To determine if any behavioural differences between SD and WKY rats are accompanied by alterations in the endocannabinoid system in the mPFC.

6.2 Materials and Methods

This study comprised of two experiments; experiment 1 and experiment 2.

6.2.1 Animals:

Experiment 1:

Male Sprague-Dawley (275 – 315g Harlan Italy) and Wister-Kyoto rats (250g – 290g; Harlan Laboratories, Belton, Loughborough, UK) were used. Before conditioning animals were housed 3 per flat bottomed cage (45 x 20 x 20cm) containing wood shavings as bedding (LBS, Surrey, UK). Animals were kept at a constant temperature ($21^{\circ}\text{C} \pm 2^{\circ}\text{C}$) under standard lighting conditions (12:12h light–dark, lights on from 0800 to 2000h). Experiments were carried out during the light phase between 0800 and 1700 h. Food (Harlan-Teklad-2014 Maintenance Diet, Harlan Laboratories, Belton, Loughborough, UK) and water were provided *ad libitum*. The experimental protocol was carried out after approval by the Animal Care and Research Ethics Committee, National University of Ireland, Galway, under license from the Health Products Regulatory Authority and in compliance with the European Communities Council directive 86/609 and 2010/63 and conformed to the ARRIVE guidelines.

Experiment 2:

Male Sprague-Dawley (270 – 310g Harlan Italy) and Wister-Kyoto rats (265g – 295g; Harlan Laboratories, Belton, Loughborough, UK) were used in this study. All other details were identical to those described for experiment 1 above.

6.2.2 Experimental procedure:

Experiment 1:

The experimental procedure was identical to that described in chapter 2 section 2.2.4 but without any microinjection.

After 30-minutes in the arena for the test trial, rats were removed and euthanised by decapitation. Brains and spinal cords were rapidly removed within a 2-minute timeframe, snap-frozen on dry ice and stored at -80°C .

This design resulted in 4 experimental groups (n=9 per group) as illustrated in Table 6.1.

Group	Conditioning	Formalin i.pl.	Strain	N
1	FC	Formalin	SD	9
2	NFC	Formalin	SD	9
3	FC	Formalin	WKY	9
4	NFC	Formalin	WKY	9

Table 6.1: Summary of experimental groups for experiment 1. i.pl., intraplantar; FC, fear-conditioned; NFC, non-fear-conditioned; SD, Sprague-Dawley; WKY, Wistar Kyoto

Experiment 2:

Identical to experiment 1 except 50µl of sterile saline was injected into the right hind paw rather than formalin. This design resulted in 4 experimental groups (n=9 per group) as illustrated in Table 6.2.

Group No.	Conditioning	Saline i.pl.	Strain	N
1	FC	Saline	SD	9
2	NFC	Saline	SD	9
3	FC	Saline	WKY	9
4	NFC	Saline	WKY	9

Table 6.2: Summary of experimental groups for experiment 2. i.pl., intraplantar; FC, fear-conditioned; NFC, non-fear-conditioned; SD, Sprague-Dawley; WKY, Wistar-Kyoto

6.2.3 Brain removal:

See chapter 2 section 2.2.5.

6.2.4 Behavioural analysis:

See chapter 2 section 2.2.6 for both experiment 1 and experiment 2.

Amendments to protocol:

Behaviour was assessed during the 10-minute fear-conditioning trial on day 1 for experiment 1. Additional behavioural data was scored during the conditioning trial. These consisted of:

1) Escape attempts: This was the amount of time the rat spent attempting to jump out of the arena.

2) Startle response: This was the time the rat spent responding to the footshock. This comprised of sudden jerking/shaking movements and excessive movement or running

6.2.5 Tissue isolation by Palkovits punch:

See chapter 3 section 3.2.8 for both experiment 1 and experiment 2.

6.2.6 Measurement of levels of endocannabinoids in the medial prefrontal cortex:

See chapter 2 section 2.2.9 with the same amendments as chapter 3 section 3.3.9 for both experiment 1 and 2.

6.2.7 Statistical analysis:

The IBM SPSS statistical software package (SPSS v23.0 for Microsoft Windows; Chicago, IL, USA) was used to analyse all data. Normality and homogeneity of variance were assessed using Shapiro-Wilk's and Levene's test, respectively. Behavioural data and mass spectrometry data were analysed by 2-way ANOVA, with fear-conditioning and drug treatment as factors. Time course behavioural data were analysed by 2-way repeated measures ANOVA with time as the within-subjects factor and fear-conditioning and strain as the between-subjects factors. Sphericity was tested using Mauchly's Test for Sphericity. If sphericity was violated a Greenhouse-Geisser correction was used. Post-hoc pairwise comparisons were made with Tukey's test when appropriate. For data that were not normally distributed, non-parametric statistics were performed. Non-parametric data was analysed using Kruskal Wallis analysis of variance and post-hoc analysis performed using Dunns-Bonferroni pairwise comparisons when appropriate. For repeated measures non-parametric data a Kruskal Wallis test was run followed by Dunn-Bonferroni post-hoc where appropriate. Data were considered significant when $p < 0.05$. Results are expressed as group means \pm standard error of the mean \pm SEM or medians with interquartile range.

6.3 Results

6.3.1 Unconditioned aversive behaviours expressed during the 10-minute fear conditioning trial in WKY and SD rats

The data examined here comprises the behaviour assessed during the 10-minute fear-conditioning trial on day 1 for experiment 1 in which rats received or did not receive footshock in the conditioning chamber.

Kruskal Wallis comparisons revealed a significant main effect on the duration of freezing ($\chi^2(3)=33.404$, $p<0.001$) over the course of the 10-minute conditioning period. Further post-hoc analysis revealed a significant increase in the duration of freezing upon fear-conditioning in both SD and WKY rats (FC SD vs. NFC SD, $p<0.001$; FC WKY vs. NFC WKY, $p<0.05$). FC WKY displayed less freezing behaviour compared to FC SD rats (FC WKY vs. FC SD, $p<0.05$) (Figure 6.1).

Kruskal Wallis comparisons revealed a significant main effect on the duration of 22kHz ultrasonic vocalisation ($\chi^2(3)=30.266$, $p<0.001$) over the course of the 10-minute conditioning period. Further post-hoc analysis revealed a significant increase in the duration of ultrasonic vocalisation upon fear-conditioning in both SD and WKY rats (FC SD vs. NFC SD, $p<0.001$; FC WKY vs. NFC WKY, $p<0.05$). FC WKY displayed less ultrasonic vocalisation compared to FC SD rats (FC WKY vs. FC SD, $p<0.05$) (Figure 6.1).

Kruskal Wallis comparisons revealed a significant main effect on the duration of escape attempts ($\chi^2(3)=20.221$, $p<0.001$) over the course of the 10-minute conditioning period. Further post-hoc analysis revealed that FC rats had an increased duration of escape attempts compared with their NFC counterparts (FC SD vs. NFC SD, $p<0.001$; FC WKY vs. NFC WKY, $p<0.05$) (Figure 6.1).

Kruskal Wallis comparisons revealed a significant main effect on the startle response duration to footshock ($\chi^2(3)=29.806$, $p<0.001$) over the course of the 10-minute conditioning period. Further post-hoc analysis revealed a significant increase in the startle response to footshock in fear-conditioned SD and WKY rats (FC SD vs. NFC SD, $p<0.001$; FC WKY vs. NFC WKY, $p<0.01$) (Figure 6.1).

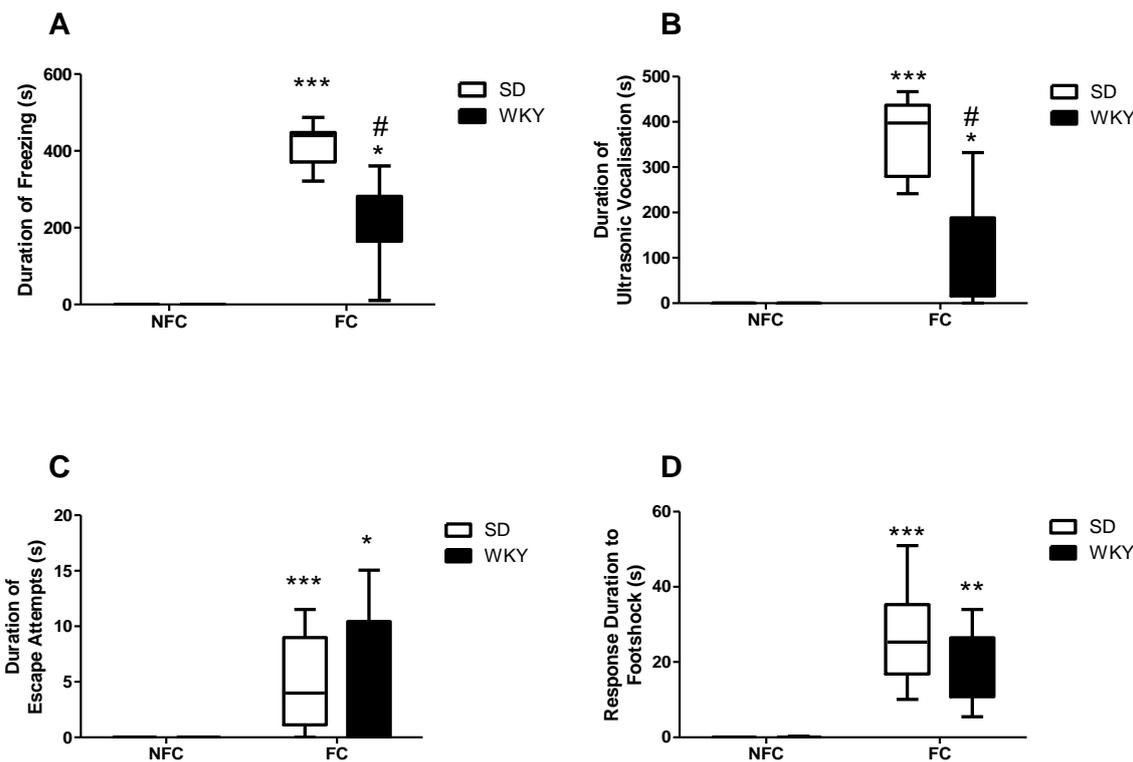


Figure 6.1: Unconditioned aversive behaviours expressed during the 10-minute fear conditioning trial in WKY and SD rats. A) duration of freezing behaviour, B) duration of 22kHz ultrasonic vocalisation, C) duration of escape attempts, D) response duration to footshock. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs NFC counterparts; # $p < 0.05$ SD vs. WKY (Dunn's-Bonferroni). All data are expressed as medians with interquartile range and min/max ($n=9$ per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned

6.3.2 General locomotor activity during the 10-minute fear conditioning trial in WKY and SD rats

Two-way ANOVA revealed a significant main effect of fear-conditioning ($F(1,32)=25.315$, $p < 0.001$) but not strain ($F(1,32)=0.522$, $p=0.475$) or fear-conditioning*strain ($F(1,32)=0.692$, $p=0.412$) on the distance moved over the course of the 10-minute conditioning period in SD and WKY rats. Further post-hoc analysis revealed a significant decrease in distance moved in both FC SD and WKY rats (FC SD vs. NFC SD, $p < 0.001$; FC WKY vs. NFC WKY, $p < 0.05$) (Table 6.3).

Two-way ANOVA revealed a significant main effect of fear-conditioning ($F(1,32)=47.946$, $p<0.001$), and fear-conditioning*strain ($F(1,32)=5.243$, $p<0.05$) but not strain ($F(1,32)=1.867$, $p=0.181$) on the duration of walking over the course of the 10-minute conditioning period in SD and WKY rats. Further post-hoc analysis revealed a significant decrease in the duration of walking in both FC SD and WKY rats (FC SD vs. NFC SD, $p<0.001$; FC WKY vs. NFC WKY, $p<0.05$) (Table 6.3).

Kruskal Wallis comparisons from k independent samples revealed a significant main effect on the duration of grooming ($\chi^2(3)=18.312$, $p<0.001$) over the course of the 10-minute conditioning period. Further post-hoc analysis revealed a significant decrease in rearing in FC SD rats compared to NFC SD (FC SD vs. NFC SD, $p<0.001$), an effect not seen in WKY rats. NFC WKY rats also displayed less rearing compared to NFC SD rats (NFC WKY vs. NFC SD, $p<0.05$) (Table 6.3).

Kruskal Wallis comparisons revealed a significant main effect on the duration of rearing ($\chi^2(3)=21.172$, $p<0.001$) over the course of the 10-minute conditioning period. Further post-hoc analysis revealed a significant decrease in rearing in FC SD rats compared to NFC SD rats (FC SD vs. NFC SD, $p<0.01$), an effect not seen in WKY rats (Table 6.3).

Group	Distance Moved (cm)	Walking (s)	Rearing (s)	Grooming (s)
FC SD	510±91***	14±3***	6(0)***	0(0)**
NFC SD	2016±382	73±12	67(31)	17(15)
FC WKY	909±185*	11±4*	35(9)#	0(0)
NFC WKY	1987±275	46±8	19(15)	6(5)

Table 6.3: Comparison of general locomotor activity and exploratory behaviours in a novel environment between WKY and SD rats, pre-formalin/saline injection. * $p<0.05$, *** $p<0.001$ vs NFC; # $p<0.05$ SD vs. WKY (Tukey's/ Dunn's-Bonferroni respectively). All data are expressed as mean \pm SEM or medians (interquartile range) (n=9 per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned

6.3.3 Nociceptive behaviour and FCA in SD and WKY rats receiving intra-plantar injection of formalin or saline

Experiment 1 - formalin:

Intra-plantar injection of formalin induced a robust nociceptive response in the form of licking, biting, shaking, flinching and elevation of the injected paw in both SD and WKY rats, in addition to right hind paw oedema (increase in hind paw diameter). Two-way ANOVA revealed no significant main effect of strain ($F(1,32)=0.023$, $p=0.882$), fear-conditioning ($F(1,32)=0.563$, $p=0.458$) or strain*fear-conditioning ($F(1,32)=0.000$, $p=1.000$) on the change in hind paw diameter pre- versus post-formalin injection (Figure 6.2).

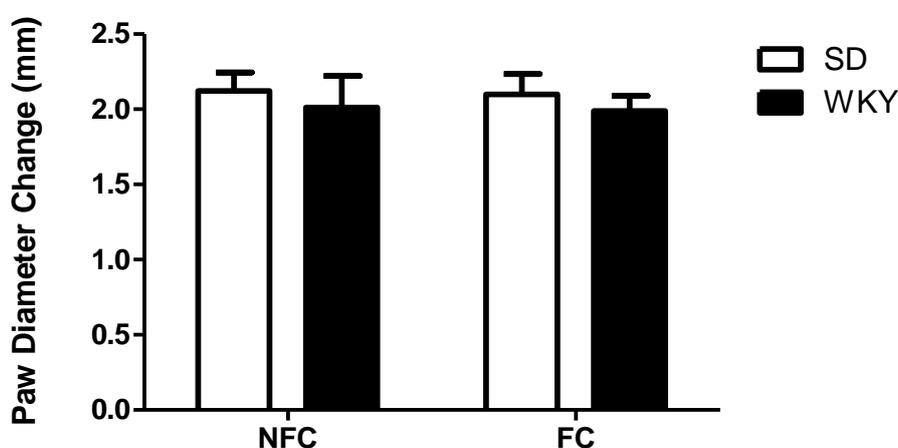


Figure 6.2: Effects of fear-conditioning on right hind paw diameter change (mm) pre and post formalin injection (oedema) in SD and WKY rats. All data are expressed as mean \pm SEM ($n=9$ per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle; SD: Sprague-Dawley; WKY: Wistar-Kyoto.

Two-way ANOVA revealed a significant main effect of fear-conditioning ($F(1,32)=8.730$, $p<0.01$) and strain ($F(1,32)=71.988$, $p<0.001$) but not fear-conditioning*strain ($F(1,32)=1.830$, $p=0.186$) on CPS over the entire 30-minute testing period. Re-exposure of SD but not WKY rats to the arena previously paired with footshock resulted in a significant reduction in formalin-evoked nociceptive behaviour, confirming the expression of FCA in SD but not WKY rats (FC SD vs. NFC SD, $p<0.05$; FC WKY vs. NFC WKY, $p=0.672$). Both NFC and FC WKY rats displayed an increased CPS over the course of the 30-minute testing period when compared

to their SD counterparts (FC WKY vs. FC SD, $p < 0.001$; NFC WKY vs. NFC SD, $p < 0.001$) (Figure 6.3).

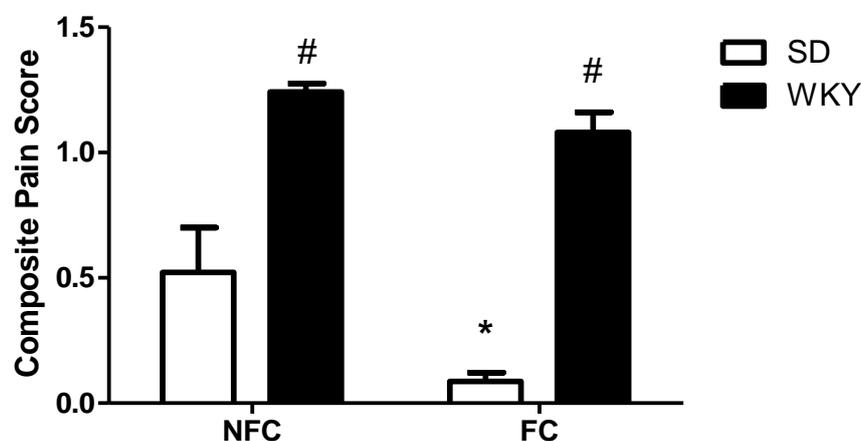


Figure 6.3: Formalin-evoked nociceptive behaviour in fear-conditioned and non-fear-conditioned SD and WKY rats over the 30-minute trial. * $p < 0.05$, FC-SD vs. NFC SD; # $p < 0.001$ WKY vs. SD (Tukey's). Data expressed as mean \pm SEM ($n = 9$ per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned

Temporal analysis of the data subdivided into 5-minute time bins using 2-way repeated measures ANOVA, revealed a significant effect of fear-conditioning ($F(1,32) = 8.730$, $p < 0.01$) and strain ($F(1,32) = 71.990$, $p < 0.001$) but not fear-conditioning*strain ($F(1,32) = 1.830$, $p = 0.186$) on CPS over the course of the 30-minute testing period. Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated ($\chi^2(14) = 47.241$, $p < 0.001$) and therefore a Greenhouse-Geisser correction was used. There was a significant main effect of time ($F(2.88,92.23) = 11.267$, $p < 0.001$), time*strain ($F(2.88,92.23) = 3.895$, $p < 0.05$) and time*fear-conditioning*strain ($F(2.88,92.23) = 3.630$, $p < 0.05$) but not time*fear-conditioning ($F(2.88,92.23) = 1.088$, $p = 0.357$). Re-exposure of SD rats to the context previously paired with footshock resulted in a significant decrease in formalin-evoked nociceptive behaviour during the middle of the testing period (between minutes 10-25 minutes), confirming the expression of FCA (FC SD vs. NFC SD; 10-15mins, 15-20mins, 20-25mins, $p < 0.05$). FC WKY rats exhibited decreased formalin-evoked nociceptive behaviour for the first 5 minutes of the trial only, confirming a transient expression of FCA in this strain (FC WKY vs. NFC WKY, 0-

5mins, $p < 0.05$). Both NFC and FC WKY rats exhibited increased CPS compared to their SD counterparts for the full 30-minute trial (At all time points: FC WKY vs. FC SD, $p < 0.001$; NFC WKY vs. NFC SD, $p < 0.001$) (Figure 6.4).

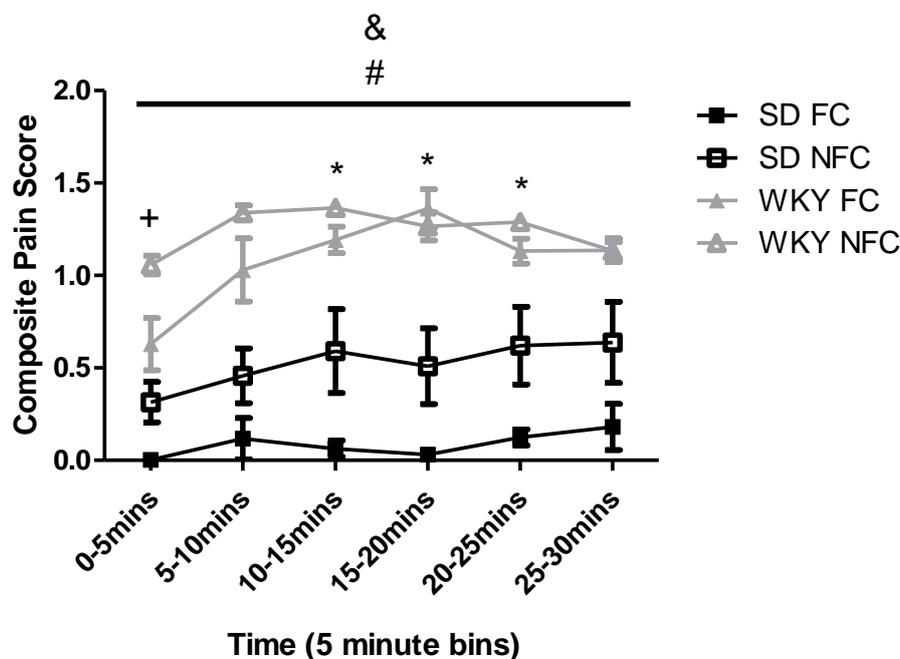


Figure 6.4: Effect of fear-conditioning on nociceptive behaviour in formalin-treated SD and WKY rats over 30-minutes in 5-minute testing bins. * $p < 0.05$, FC SD vs. NFC SD; + $p < 0.05$, FC WKY vs. NFC WKY; # $p < 0.001$, FC SD vs. FC WKY; & $p < 0.001$, NFC SD vs. NFC WKY (Tukey's). All data are expressed as mean \pm SEM ($n = 9$ per group) SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned.

Experiment 2 - saline:

Unlike formalin, intra-plantar injection of saline did not induce robust licking, biting, shaking and elevation or oedema of the injected paw in either SD or WKY rats. Nevertheless, for comparison with experiment 1, CPS was calculated, analysed and graphed. Two-way ANOVA revealed a significant main effect of fear-conditioning ($F(1,32) = 14.362$, $p < 0.001$), strain ($F(1,32) = 11.923$, $p < 0.01$) and fear-conditioning*strain ($F(1,32) = 16.625$, $p < 0.001$) on CPS over the entire 30-minute testing period. Re-exposure of SD but not WKY rats to the arena previously paired with footshock resulted in a significant reduction in CPS in SD but not WKY

rats (FC SD vs. NFC SD, $p < 0.001$; FC WKY vs. NFC WKY, $p = 0.997$). FC WKY rats displayed an increased CPS over the course of the 30-minute testing period when compared to their SD counterparts (FC WKY vs. FC SD, $p < 0.001$) (Figure 6.5).

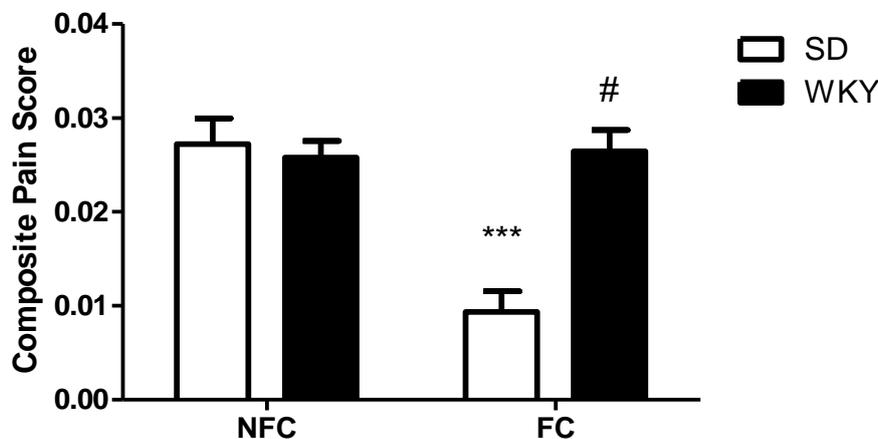


Figure 6.5: Composite pain scores in fear-conditioned and non-fear-conditioned SD and WKY rats receiving intra-plantar saline over the 30-minute trial. *** $p < 0.001$, FC-SD vs. NFC SD; # $p < 0.001$ WKY vs. SD (Tukey's). Data expressed as mean \pm SEM ($n = 9$ per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned.

Temporal analysis of the data subdivided into 5-minute time bins using 2-way repeated measures ANOVA, revealed a significant effect of fear-conditioning ($F(1,32) = 14.362$, $p < 0.001$), strain ($F(1,32) = 11.923$, $p < 0.01$) and fear-conditioning*strain ($F(1,32) = 16.625$, $p < 0.001$) on CPS. There was also a significant main effect of time ($F(5,160) = 2.572$, $p < 0.05$), time*fear-conditioning ($F(5,160) = 18.128$, $p < 0.001$), time*strain ($F(5,160) = 3.388$, $p < 0.01$) and time*fear-conditioning*strain ($F(5,160) = 2.456$, $p < 0.05$). Post-hoc analysis revealed a decreased CPS for the first 20 minutes of the testing period in fear-conditioned SD rats (FC SD vs. NFC SD, 0-5mins, 5-10mins, 10-15mins, $p < 0.001$; 15-20mins, $p < 0.05$). FC WKY rats displayed a lower CPS for the first 5 minutes of the trial only (FC WKY vs. NFC WKY, 0-5 min, $p < 0.001$). FC WKY rats display increased CPS compared to their SD counterparts between minutes 5-20 (FC WKY vs. FC SD, 5-10mins, 10-15mins, 15-20mins $p < 0.001$) (Figure 6.6).

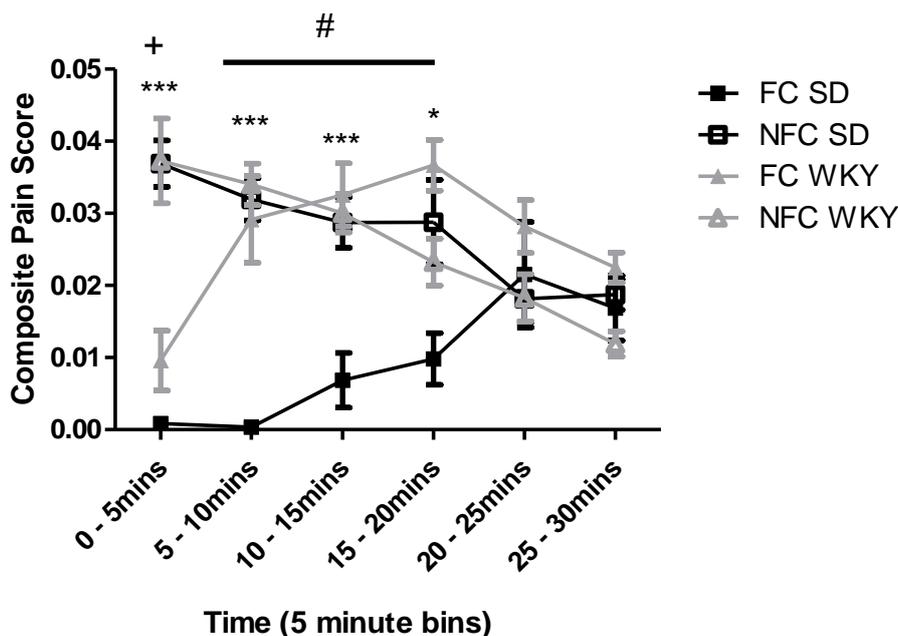


Figure 6.6: Effect of fear-conditioning on CPS in intra-plantar saline-treated SD and WKY rats over 30-minutes in 5-minute testing bins. * $p < 0.05$, *** $p < 0.001$, FC SD vs. NFC SD; + $p < 0.05$, FC WKY vs. NFC WKY; # $p < 0.001$, FC SD vs, FC WKY (Tukey's). All data are expressed as mean \pm SEM ($n = 9$ per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned.

6.3.4 Effect of strain on expression of conditioned fear behaviour in the presence and absence of formalin-evoked nociceptive tone

Experiment 1 - formalin:

Duration of Freezing:

Kruskal Wallis comparisons revealed a significant main effect on the duration of freezing ($\chi^2(3) = 26.680$, $p < 0.001$) over the course of the 30-minute testing period. Post-hoc analysis revealed that FC SD and WKY rats displayed robust freezing behaviour over the course of the 30-minute trial compared to their NFC counterparts (FC SD vs. NFC SD, $p < 0.001$; FC WKY vs. NFC WKY, $p < 0.05$). FC SD rats displayed a significantly greater duration of freezing behaviour over the course of the 30-minute trial compared to fear-conditioned WKY rats (FC WKY vs. FC SD, $p < 0.001$), an effect not seen in NFC rats (Figure 6.7).

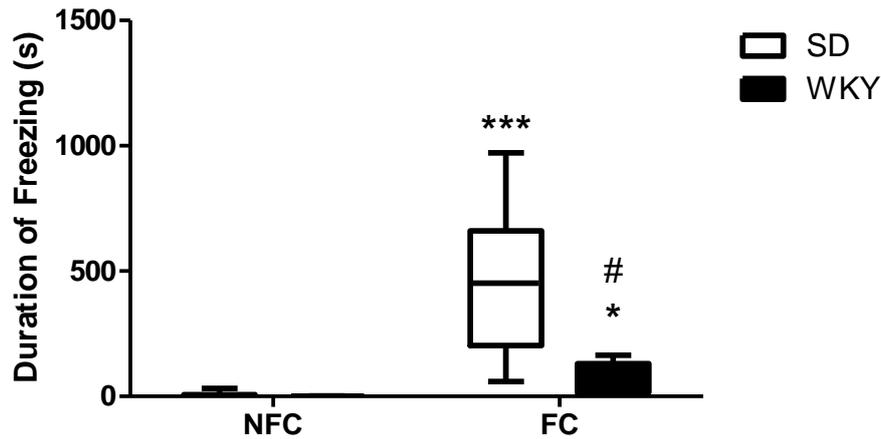


Figure 6.7: Effect of fear-conditioning on freezing behaviour in formalin-treated SD and WKY rats. * $p < 0.05$, *** $p < 0.001$, FC vs. NFC; # $p < 0.001$ FC-SD vs. FC-WKY (Dunn's-Bonferroni). All data are expressed as medians with interquartile range ($n=9$ per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned.

Analysis of the data subdivided into 5-minute time bins using Kruskal-Wallis revealed a significant between-group effect on the expression of freezing at 0-5mins ($\chi^2(3)=29.746$, $p < 0.001$), 5-10mins ($\chi^2(3)=23.910$, $p < 0.001$) and 10-15mins ($\chi^2(3)=10.437$, $p < 0.05$) but not 15-20mins ($\chi^2(3)=2.589$, $p=0.459$), 20-25mins, ($\chi^2(3)=4.518$, $p=0.211$) or 25-30mins ($\chi^2(3)=3.000$, $p=0.392$). Post-hoc analysis revealed the duration of freezing was increased in the first 10-minutes of the trial in FC SD rats (FC SD vs. NFC SD, 0-5mins, 5-10mins $p < 0.001$) and in the first five minutes of the testing period in FC WKY rats (0-5mins FC WKY vs. NFC WKY $p < 0.05$). FC SD rats also display increased freezing when compared to FC WKY rats in the first 10-minutes of the trial (FC WKY vs. FC SD, 0-5mins, 5-10mins, $p < 0.001$) (Figure 6.8).

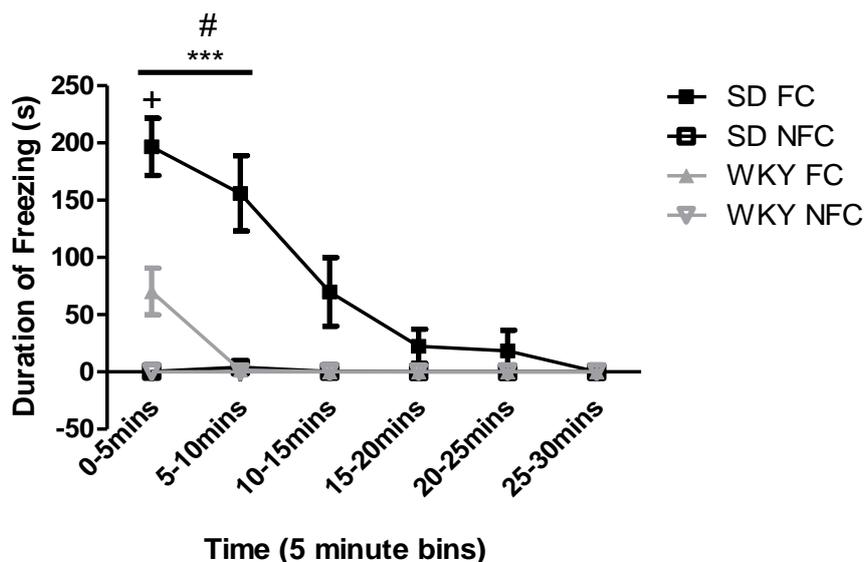


Figure 6.8: Effect of fear-conditioning on freezing behaviour in formalin-treated SD and WKY rats over the 30-minute trial in 5-minute testing bins. *** $p < 0.001$, FC SD vs. NFC SD; + $p < 0.05$, FC WKY vs. NFC WKY; # $p < 0.001$, FC SD vs, FC WKY (Dunn's-Bonferroni). All data are expressed as mean \pm SEM ($n=9$ per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned.

Duration of 22kHz Ultrasonic Vocalisation:

Kruskal Wallis comparisons from k independent samples revealed a significant main effect on the duration of 22kHz ultrasonic vocalisation ($\chi^2(3)=13.413$, $p < 0.01$) over the course of the 30-minute testing period. Post-hoc analysis revealed that FC SD but not WKY rats expressed increased 22kHz ultrasonic vocalization over the course of the 30-minute testing period compared to their NFC counterparts (FC SD vs. NFC SD, $p < 0.05$). FC SD rats displayed increased 22kHz ultrasonic vocalization over the course of the 30-minute trial compared to FC WKY rats (FC WKY vs. FC SD, $p < 0.05$), an effect not seen in NFC rats (Figure 6.9).

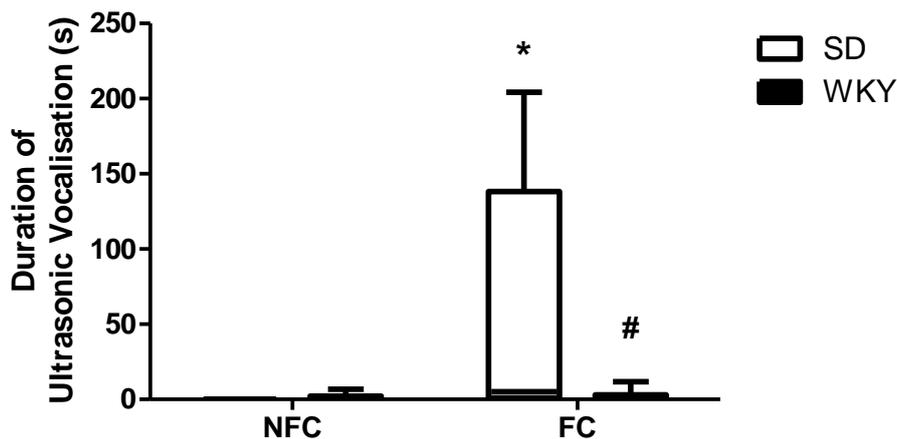


Figure 6.9: Effect of fear-conditioning on 22kHz ultrasonic vocalisation in formalin-treated SD and WKY rats over the 30-minute trial. NFC-SD vs. FC-SD, * $p < 0.05$. FC-SD vs. FC WKY, # $p < 0.05$ (Dunn's-Bonferroni). All data are expressed as medians with interquartile range ($n = 9$ per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned.

Analysis of the data subdivided into 5-minute time bins using Kruskal-Wallis revealed a significant between-group effect on the expression of freezing at 0-5mins ($\chi^2(3) = 8.464$, $p < 0.05$), 5-10mins ($\chi^2(3) = 17.981$, $p < 0.001$) and 10-15mins ($\chi^2(3) = 9.811$, $p < 0.05$) but not 15-20mins ($\chi^2(3) = 4.385$, $p = 0.223$), 20-25mins, ($\chi^2(3) = 2.060$, $p = 0.560$) or 25-30mins ($\chi^2(3) = 3.000$, $p = 0.392$). Post-hoc analysis revealed no significant differences between the groups (Figure 6.10).

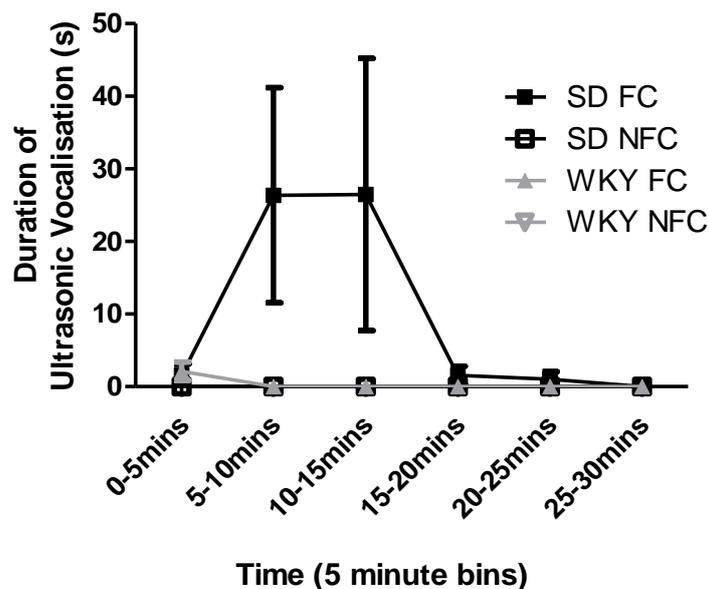


Figure 6.10: Effect of fear-conditioning on 22kHz ultrasonic vocalisation in formalin-treated SD and WKY rats over the 30-minute trial in 5-minute testing bins. All data are expressed as mean \pm SEM (n=9 per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned.

Experiment 2 - saline:

Duration of Freezing:

Kruskal Wallis comparisons from k independent samples revealed a significant main effect on the duration of freezing ($\chi^2(3)=33.765$, $p<0.001$) over the course of the 30-minute testing period. Re-exposure to a context previously paired with an aversive stimulus increased the duration of freezing in both SD and WKY rats (FC SD vs. NFC SD, $p<0.001$; FC WKY vs. NFC WKY, $p<0.05$). FC SD displayed increased freezing behaviour over the course of the 30-minute trial compared to fear-conditioned WKY rats (FC WKY vs. FC SD, $p<0.05$) (Figure 6.11).

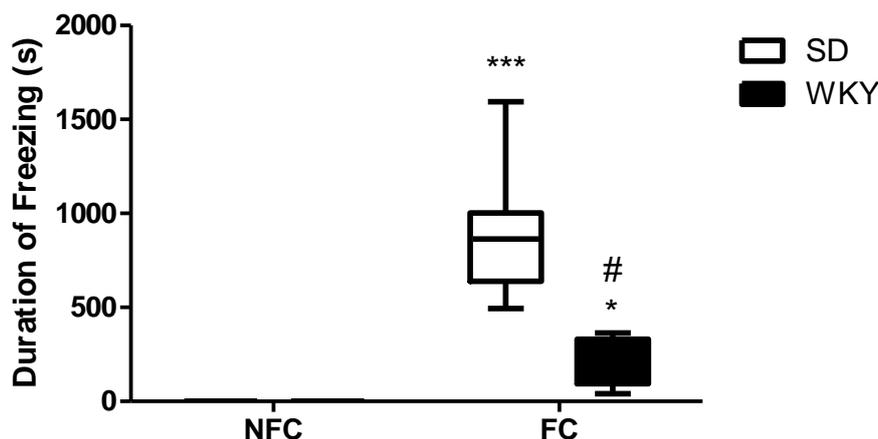


Figure 6.11: Effect of fear-conditioning on freezing behaviour in intra-plantar saline-treated SD and WKY rats over 30-minutes. FC-SD vs. NFC-SD. *** $p < 0.001$; FC-SD vs. FC-WKY, # $p < 0.001$ (Dunn's-Bonferroni). All data are expressed as medians with interquartile range ($n=9$ per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned.

Analysis of the data subdivided into 5-minute time bins using Kruskal-Wallis revealed a significant between-group effect on the expression of freezing at 0-5mins ($\chi^2(3)=31.619$, $p < 0.001$), 5-10mins ($\chi^2(3)=30.130$, $p < 0.001$), 10-15mins ($\chi^2(3)=29.395$, $p < 0.001$), 15-20mins ($\chi^2(3)=20.776$, $p < 0.001$) and 20-25mins, ($\chi^2(3)=9.347$, $p < 0.05$) but not 25-30mins ($\chi^2(3)=2.060$, $p=0.560$). Post-hoc analysis revealed a significant increase in the duration of freezing in the first 20 minutes of the trial in FC SD rats (FC SD vs NFC SD, 0-5mins, 5-10mins, 10-15mins, $p < 0.001$; 15-20mins $p < 0.05$) and in the first 5 minutes of the testing period in FC WKY rats (FC WKY vs. NFC WKY, 0-5 mins, $p < 0.001$). FC SD rats also display an increased duration of freezing when compared to FC WKY rats in the first 20 minutes of the trial (FC WKY vs. FC SD, 0-5mins, 5-10mins, 10-15mins, $p < 0.001$; 15-20mins $p < 0.05$) an effect not seen in NFC rats (Figure 6.12).

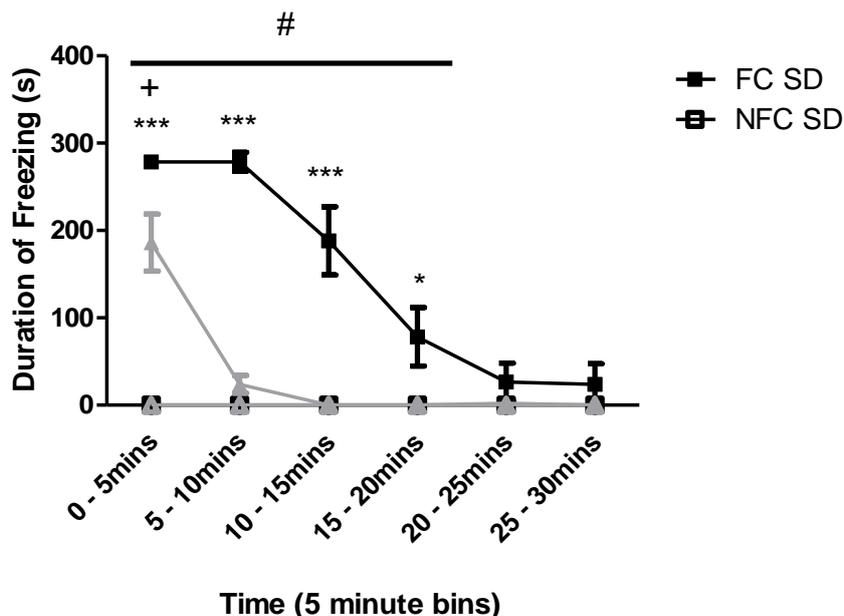


Figure 6.12: Effect of fear-conditioning on freezing behaviour in intra-plantar saline-treated SD and WKY rats over 30-minutes in 5-minute testing bins. * $p < 0.05$, *** $p < 0.001$, FC SD vs. NFC SD; + $p < 0.05$, FC WKY vs. NFC WKY; # $p < 0.001$, FC SD vs. FC WKY (Dunn's-Bonferroni). All data are expressed as mean \pm SEM ($n=9$ per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned.

Duration of 22kHz Ultrasonic Vocalisation:

Kruskal Wallis comparisons from k independent samples revealed a significant main effect on the duration of 22kHz ultrasonic vocalisation ($\chi^2(3)=13.068$, $p < 0.01$) over the course of the 30-minute testing period. Post-hoc analysis revealed that re-exposure to the arena previously paired with footshock significantly increased the duration of 22kHz ultrasonic vocalisation in FC SD but not WKY rats (FC SD vs. NFC SD, $p < 0.05$). FC SD rats displayed significantly more ultrasonic vocalisation compared to FC WKY rats (FC WKY vs. FC SD, $p < 0.05$) (Figure 6.13).

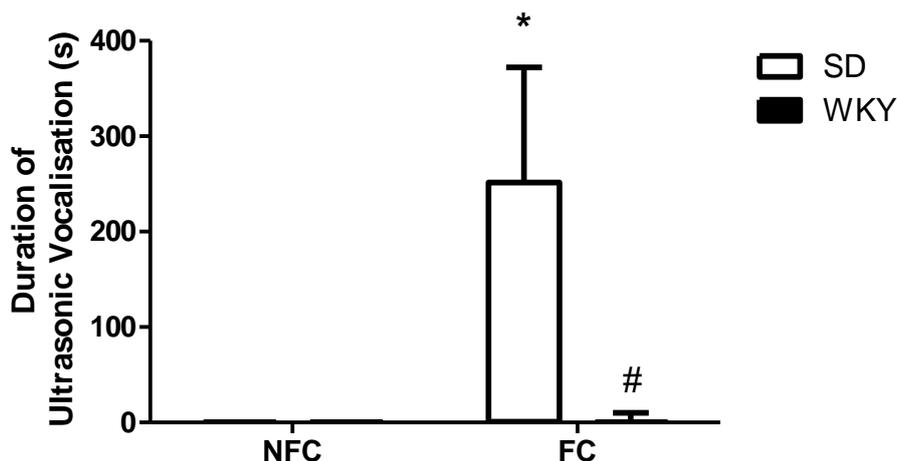


Figure 6.13: Effect of fear-conditioning on 22kHz ultrasonic vocalisation in intra-plantar saline-treated SD and WKY rats over 30-minutes. * $p < 0.05$ vs NFC, # $p < 0.05$ (Dunn's-Bonferroni). All data are expressed as medians with interquartile range ($n=9$ per group). FC SD vs. FC WKY. SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned.

Analysis of the data subdivided into 5-minute time bins using Kruskal-Wallis revealed a significant between-group effect on the expression of freezing at 5-10mins ($\chi^2(3)=9.524$, $p < 0.05$) and 10-15mins ($\chi^2(3)=16.815$, $p < 0.001$) but not 0-5mins ($\chi^2(3)=4.406$, $p < 0.257$), 15-20mins ($\chi^2(3)=3.000$, $p=0.392$), 20-25mins, ($\chi^2(3)=2.060$, $p=0.560$) or 25-30mins ($\chi^2(3)=0.000$, $p=1.000$). Post-hoc analysis revealed no significant differences between the groups (Figure 6.14).

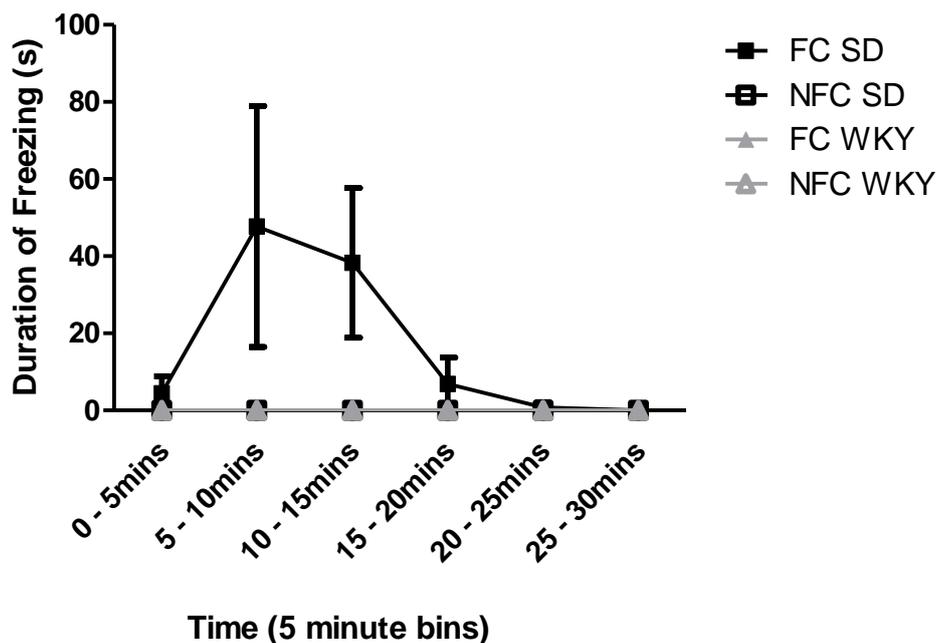


Figure 6.14: Effect of fear-conditioning on 22kHz ultrasonic vocalisation in intra-plantar saline-treated SD and WKY rats over 30-minutes in 5-minute testing bins. All data are expressed as mean \pm SEM ($n=9$ per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned.

6.3.5 Effects of fear-conditioning and strain on general locomotor behaviours and defecation in formalin or saline treated rats

Experiment 1:

Two-way ANOVA revealed a significant main effect of strain ($F(1,32)=20.882$, $p<0.01$) and strain*fear-conditioning ($F(1,32)=6.494$, $p<0.05$) but not fear-conditioning ($F(1,32)=1.176$, $p=0.286$) on the distance moved over the course of the 30-minute testing period. Post-hoc analysis revealed that NFC SD rats (NFC SD vs. NFC WKY, $p<0.001$) moved a greater distance compared to non-fear-conditioned WKY rats (Table 6.4).

Two-way ANOVA revealed a significant main effect of strain ($F(1,32)=10.882$, $p<0.01$) and strain*fear-conditioning ($F(1,32)=6.494$, $p<0.05$) but not fear conditioning ($F(1,32)=1.176$, $p=0.286$) for the amount of time spent walking over the 30-minute testing period. Further post-hoc analysis revealed that NFC SD rats (NFC WKY vs. NFC SD, $p<0.05$) showed increased walking compared to non-fear-conditioned WKY rats (Table 6.4).

Two-way ANOVA revealed a significant main effect of strain ($F(1,32)=12.987$, $p<0.001$) but not fear-conditioning ($F(1,32)=0.267$, $p=0.609$) or strain*fear-conditioning ($F(1,32)=0.808$, $p=0.375$) on the amount of time spent rearing in the arena over the 30-minute testing period. Further post-hoc analysis showed that NFC SD rats (NFC WKY vs. NFC SD, $p<0.05$) reared more than NFC WKY rats (Table 6.4).

Two-way ANOVA revealed a significant main effect of strain ($F(1,32)=10.564$, $p<0.01$) but not fear-conditioning ($F(1,32)=1.477$, $p=0.233$) or strain*fear-conditioning ($F(1,32)=0.467$, $p=0.499$) on the amount of time spent grooming in the arena over the 30-minute testing period. Further Post-hoc analysis showed that NFC SD (NFC WKY vs. NFC SD, $p<0.05$) rats groomed significantly more than NFC WKY rats (Table 6.4).

Kruskal Wallis comparisons revealed a significant effect ($\chi^2(3)=14.433$, $p<0.01$) for the amount of defecation in the arena (no. of pellets) over the course of the 30-minute testing period. Further post-hoc analysis revealed that FC SD but not WKY rats (FC SD vs. NFC SD, $p<0.01$) exhibited a significant increase in defecation over the course of the 30-minute testing period compared to their NFC counterparts. FC SD rats defecated more than FC WKY rats (FC WKY vs. FC SD, $p<0.05$), an effect not seen in NFC rats (Table 6.4).

Groups	Distance Moved (cm)	Walking (s)	Rearing (s)	Grooming (s)	Defecation (number of pellets)
FC SD	4104±590	118±30	21±9	57±18	7(6)***
NFC SD	5618±447	196±21	31±10	88±25	0(3)
FC WKY	3791±284	87±15	3±2	15±4	1(4)#
NFC WKY	3181±257#	101±13#	0.4±0.2#	24±10#	1(3)

Table 6.4: Effect of fear-conditioning on distance moved, walking, rearing, grooming, and defecation in formalin-treated SD and WKY rats. *** $p<0.001$ vs NFC counterparts, # $p<0.05$ SD vs. WKY (Tukey's/Dunn's-Bonferroni respectively). All data are expressed as mean \pm SEM or medians (interquartile range) ($n=9$ per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned.

Experiment 2:

Two-way ANOVA revealed a significant main effect of fear-conditioning ($F(1,32)=14.885$, $p<0.001$) and strain*fear-conditioning ($F(1,32)=13.658$, $p<0.001$) but not strain on distance moved ($F(1,32)=0.034$, $p=0.856$). Further Post-hoc analysis revealed that FC SD rats moved less than both NFC SD and FC WKY rats (FC SD vs NFC SD, $p<0.001$; FC WKY vs. FC SD, $p<0.05$) (Table 6.5).

Two-way ANOVA revealed a significant main effect of fear conditioning ($F(1,32)=11.362$, $p<0.01$), strain ($F(1,32)=11.478$, $p<0.01$) and strain*fear-conditioning ($F(1,32)=22.523$, $p<0.001$) on the amount of time spent walking over the 30-minute testing period. Further Post-hoc analysis revealed that FC SD rats walked less than both NFC SD and FC WKY rats (FC SD vs NFC SD, $p<0.001$; FC WKY vs. FC SD, $p<0.001$) (Table 6.5).

Two-way ANOVA revealed a significant main effect of strain ($F(1,32)=29.457$, $p<0.001$) but not fear-conditioning ($F(1,32)=0.327$, $p=0.571$) or strain*fear-conditioning ($F(1,32)=2.741$, $p=0.108$) on the amount of time spent rearing in the arena over the 30-minute testing period. Further Post-hoc analysis revealed that NFC WKY rats reared less than NFC SD rats (NFC WKY vs. NFC SD $p<0.001$) (Table 6.5).

Two-way ANOVA revealed a significant main effect of fear-conditioning ($F(1,32)=11.252$, $p<0.05$) but not strain ($F(1,32)=2.877$, $p=0.100$) or strain*fear-conditioning ($F(1,32)=0.001$, $p=0.975$) on the amount of time spent grooming in the arena over the 30-minute testing period. Further Post-hoc analysis revealed no significant effects (Table 6.5).

Kruskal Wallis comparisons revealed a significant effect ($\chi^2(3)=20.412$, $p<0.001$) for the amount of defecation in the arena (no. of pellets) over the course of the 30-minute testing period. Further post-hoc analysis revealed that FC SD but not WKY rats (FC SD vs. NFC SD, $p<0.01$) exhibited a significant increase in defecation over the course of the 30-minute testing period compared to their NFC counterparts. FC SD rats defecated more than FC WKY rats (FC WKY vs. FC SD, $p<0.001$), an effect not seen in NFC rats (Table 6.5).

Groups	Distance Moved (cm)	Walking (s)	Rearing (s)	Grooming (s)	Defecation (number of pellets)
FC SD	2143±330***	37±8***	97±20	39±12	6&3***
NFC SD	4033±308	131±15	130±20	97±17	2&2
FC WKY	3113±152#	131±12#	41±8	68±7	2&2#
NFC WKY	3154±154	115±10	25±5#	127±28	2&2

Table 6.5: Effect of fear-conditioning and strain on distance moved, walking, rearing, grooming, paw oedema and defecation in saline treated SD and WKY rats. *** $p < 0.001$ vs NFC counterparts; # $p < 0.05$ SD vs WKY (Tukey's/Dunn's-Bonferroni respectively). All data are expressed as mean \pm SEM or medians with interquartile range (n=9 per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned.

6.3.6 Effect of fear-conditioning and strain on AEA, OEA, PEA or 2-AG levels in the IL, PrL and ACC of formalin-treated rats

Experiment 1:

Infralimbic Cortex

Two-way ANOVA revealed no significant main effect of fear conditioning ($F(1,28)=0.208$, $p=0.652$), or strain*fear-conditioning ($F(1,28)=0.237$, $p=0.630$) but did reveal a significant main effect of strain ($F(1,28)=4.178$, $p < 0.05$) on AEA in the IL. Post-hoc analysis revealed no significant difference between SD and WKY rats (Figure 6.15).

Two-way ANOVA revealed no significant main effect of fear conditioning ($F(1,28)=1.543$, $p=0.275$), strain ($F(1,28)=0.558$, $p=0.461$), or strain*fear-conditioning ($F(1,28)=0.905$, $p=0.349$) on 2-AG in the IL (Figure 6.15).

Two-way repeated measures ANOVA revealed no significant main effect of fear conditioning ($F(1,28)=1.238$, $p=0.224$), strain ($F(1,28)=3.615$, $p=0.068$), or strain*fear-conditioning ($F(1,28)=0.151$, $p=0.700$) on PEA in the IL (Figure 6.15).

Two-way repeated measures ANOVA revealed no significant main effect of fear conditioning ($F(1,29)=0.723$, $p=0.402$), strain ($F(1,29)=0.972$, $p=0.332$), or strain*fear-conditioning ($F(1,29)=0.003$, $p=0.958$) on OEA in the IL (Figure 6.15).

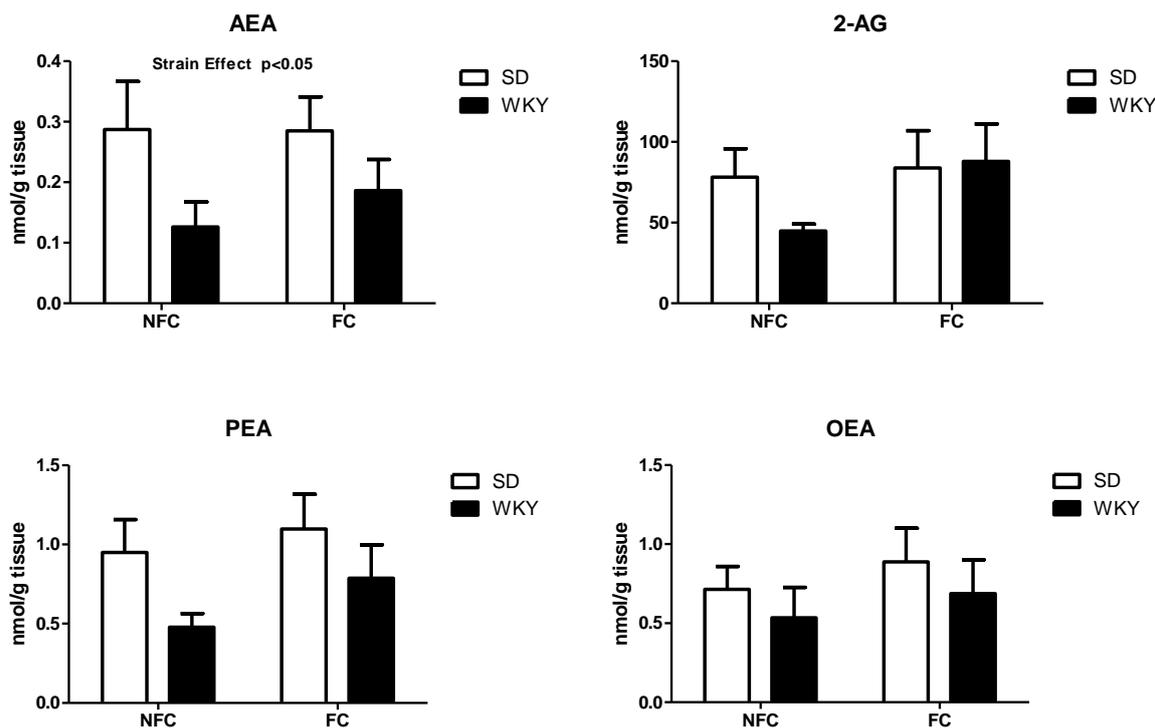


Figure 6.15: Effect of fear-conditioning and strain on AEA, OEA, PEA or 2-AG levels in the IL of formalin-treated rats. All data are expressed as mean ± SEM (n=6-9 per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned.

Prelimbic Cortex

Two-way ANOVA revealed no significant main effect of fear conditioning ($F(1,28)=0.020$, $p=0.889$), strain ($F(1,28)=0.832$, $p=0.369$), or strain*fear-conditioning ($F(1,28)=0.285$, $p=0.996$) on AEA in the PrL (Figure 6.16).

Two-way ANOVA revealed no significant main effect of fear conditioning ($F(1,27)=0.012$, $p=0.915$), strain ($F(1,27)=0.019$, $p=0.893$), or strain*fear-conditioning ($F(1,27)=0.280$, $p=0.601$) on 2-AG in the PrL (Figure 6.16).

Two-way repeated measures ANOVA revealed no significant main effect of fear conditioning ($F(1,26)=0.003$, $p=0.960$), strain ($F(1,26)=0.008$, $p=0.928$), or strain*fear-conditioning ($F(1,26)=0.820$, $p=0.378$) on PEA in the PrL (Figure 6.16).

Two-way repeated measures ANOVA revealed no significant main effect of fear conditioning ($F(1,27)=0.409$, $p=0.528$), strain ($F(1,27)=0.902$, $p=0.350$), or strain*fear-conditioning ($F(1,27)=0.123$, $p=0.728$) on OEA in the PrL (Figure 6.16).

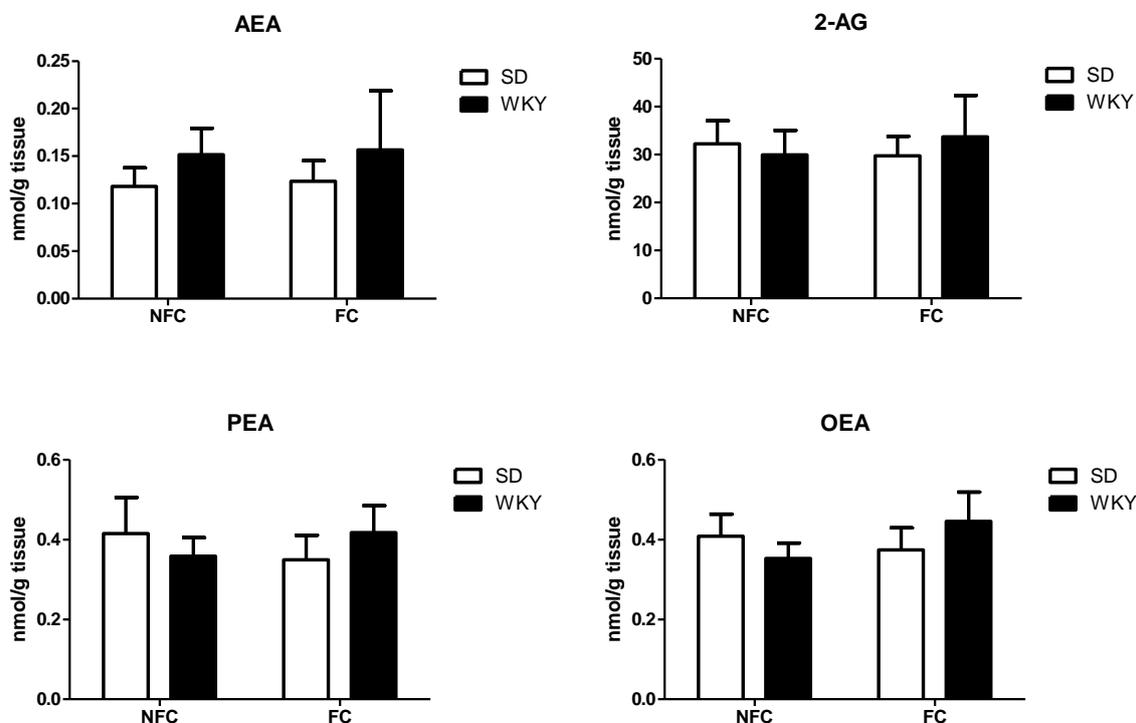


Figure 6.16: Effect of fear-conditioning and strain on AEA, OEA, PEA or 2-AG levels in the PrL of formalin-treated rats. All data are expressed as mean \pm SEM ($n=7-9$ per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned.

Anterior Cingulate Cortex

Two-way ANOVA revealed no significant main effect of fear conditioning ($F(1,28)=0.102$, $p=0.751$), strain ($F(1,28)=0.057$, $p=0.812$), or strain*fear-conditioning ($F(1,28)=0.241$, $p=0.627$) on AEA in the ACC (Figure 6.17).

Two-way ANOVA revealed no significant main effect of fear conditioning ($F(1,29)=0.004$, $p=0.951$), strain ($F(1,29)=0.214$, $p=0.647$), or strain*fear-conditioning ($F(1,29)=0.015$, $p=0.904$) on 2-AG in the ACC (Figure 6.17).

Two-way ANOVA revealed no significant main effect of fear conditioning ($F(1,32)=0.462$, $p=0.502$), strain ($F(1,32)=2.463$, $p=0.127$), or strain*fear-conditioning ($F(1,32)=0.163$, $p=0.689$) on PEA in the ACC (Figure 6.17).

Two-way repeated measures ANOVA revealed no significant main effect of fear conditioning ($F(1,28)=0.308$, $p=0.583$), strain ($F(1,28)=1.292$, $p=0.265$), or strain*fear-conditioning ($F(1,28)=1.084$, $p=0.306$) on OEA in the ACC (Figure 6.17).

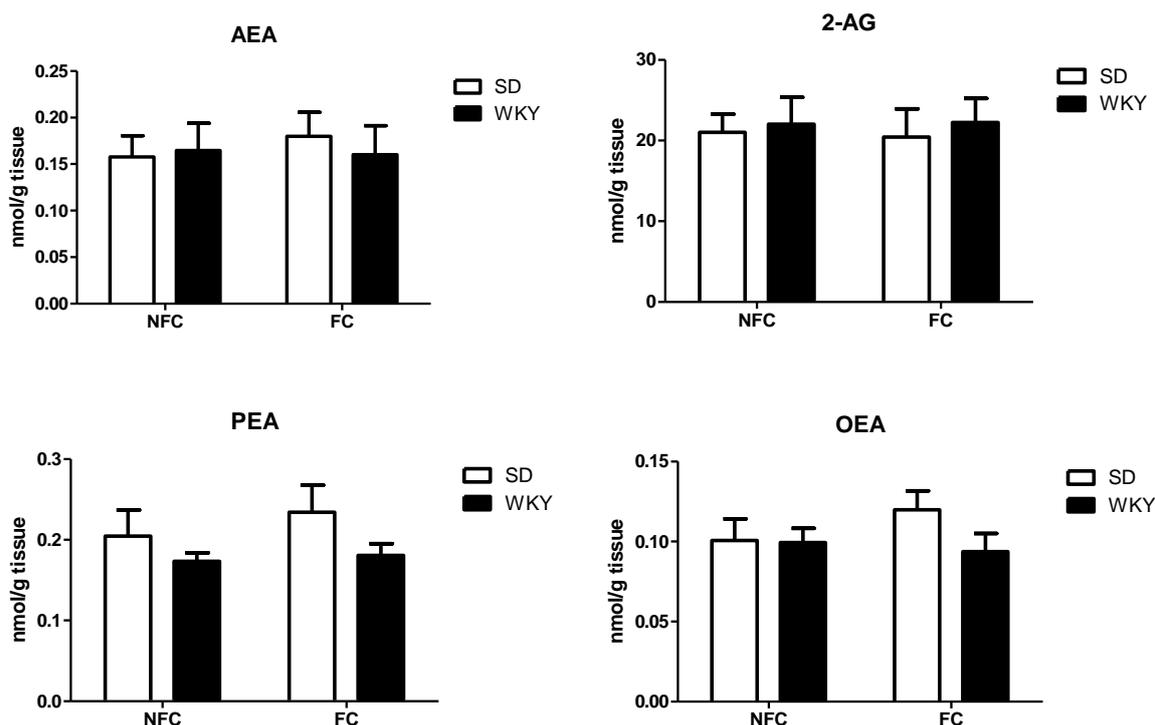


Figure 6.17: Effect of fear-conditioning and strain on AEA, OEA, PEA or 2-AG levels in the ACC of formalin-treated rats. All data are expressed as mean \pm SEM ($n=7-9$ per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned.

Experiment 2:

Infralimbic Cortex:

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,30)=0.057$, $p=0.812$), strain ($F(1,30)=1.089$, $p=0.305$) or fear-conditioning*strain ($F(1,30)=0.961$, $p=0.335$) on the levels of AEA in the IL of SD and WKY rats (Figure 6.18).

Two-way ANOVA revealed a significant effect of strain ($F(1,30)=6.902$, $p<0.05$) but not fear-conditioning ($F(1,30)=0.176$, $p=0.678$) or fear-conditioning*strain ($F(1,30)=0.205$, $p=0.654$) on the levels of 2-AG in the IL of SD and WKY rats. Further Post-hoc analysis revealed no significant effects (Figure 6.18).

Two-way ANOVA revealed a significant effect of strain ($F(1,29)=9.813$, $p<0.01$) but not fear-conditioning ($F(1,29)=0.287$, $p=0.596$) or fear-conditioning*strain ($F(1,29)=0.011$, $p=0.917$) on the levels of PEA in the IL of SD and WKY rats. Further Post-hoc analysis revealed no significant effects (Figure 6.18).

Two-way ANOVA revealed a significant effect of strain ($F(1,29)=6.435$, $p<0.05$) but not fear-conditioning ($F(1,29)=0.125$, $p=0.726$) or fear-conditioning*strain ($F(1,29)=0.367$, $p=0.549$) on the levels of OEA in the IL of SD and WKY rats. Further Post-hoc analysis revealed no significant effects (Figure 6.18).

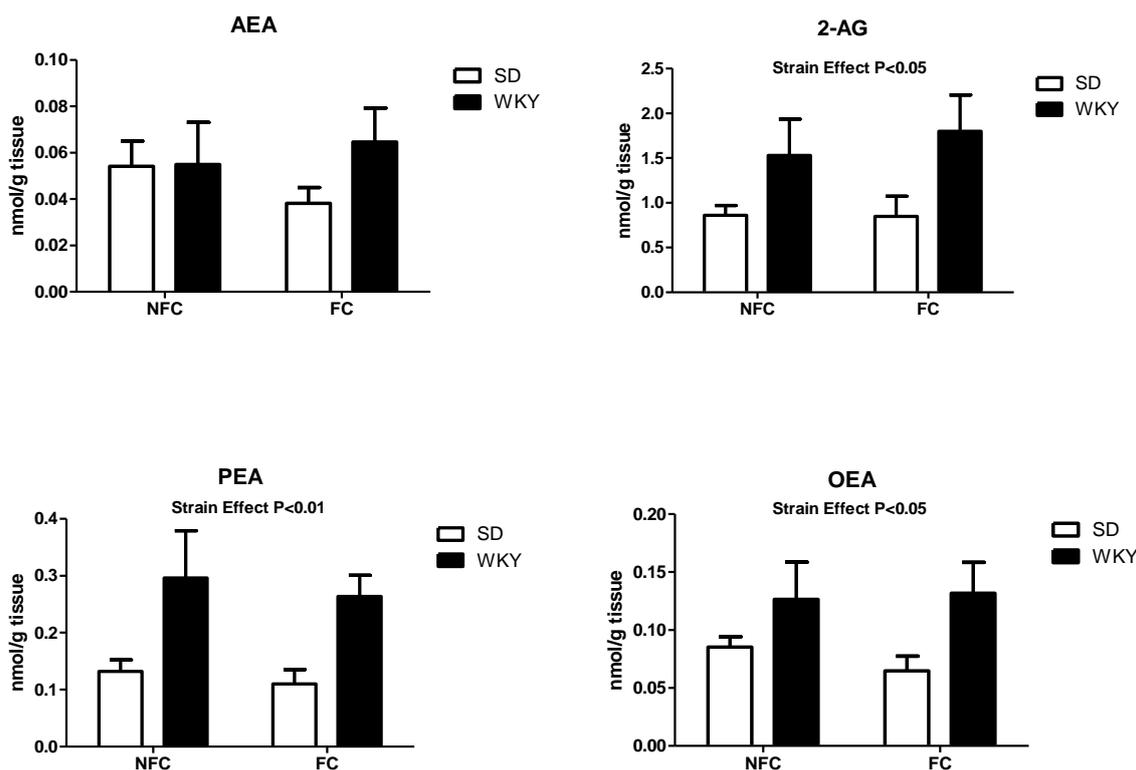


Figure 6.18: Effect of fear-conditioning and strain on AEA, OEA, PEA or 2-AG levels in the IL of saline-treated rats. All data are expressed as mean \pm SEM ($n=8-9$ per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned.

Prelimbic Cortex:

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,29)=0.716$, $p=0.404$), strain ($F(1,29)=0.379$, $p=0.543$) or fear-conditioning*strain ($F(1,29)=0.929$, $p=0.343$) on the levels of AEA in the PrL of SD and WKY rats (Figure 6.19).

Two-way ANOVA revealed a significant effect of strain ($F(1,29)=4.379$, $p<0.05$) but not fear-conditioning ($F(1,29)=2.047$, $p=0.163$) or fear-conditioning*strain ($F(1,29)=0.550$, $p=0.464$) on the levels of 2-AG in the PrL of SD and WKY rats. Further Post-hoc analysis revealed no significant effects (Figure 6.19).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,30)=0.234$, $p=0.636$), strain ($F(1,30)=1.930$, $p=0.175$) or fear-conditioning*strain ($F(1,30)=1.411$, $p=0.244$) on the levels of PEA in the PrL of SD and WKY rats (Figure 6.19).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,28)=1.721$, $p=0.200$), strain ($F(1,28)=1.142$, $p=0.294$) or fear-conditioning*strain ($F(1,28)=1.908$, $p=0.178$) on the levels of OEA in the PrL of SD and WKY rats (Figure 6.19).

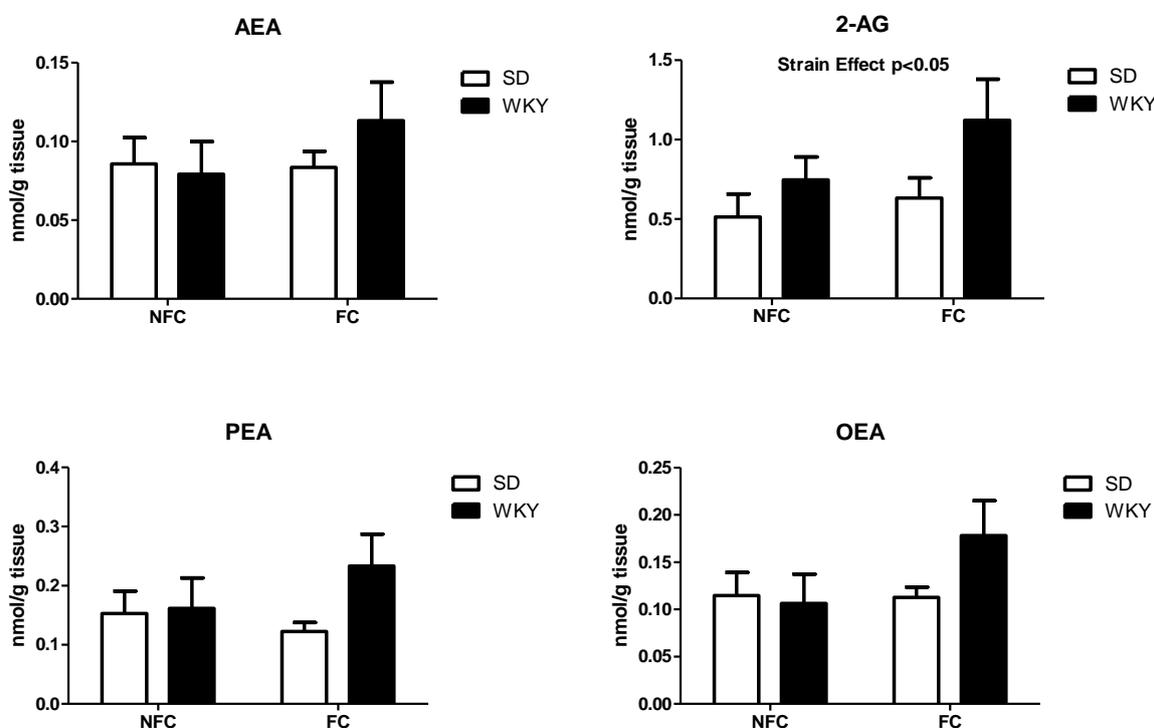


Figure 6.19: Effect of fear-conditioning and strain on AEA, OEA, PEA or 2-AG levels in the PrL of saline-treated rats. All data are expressed as mean \pm SEM (n=7-9 per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned.

Anterior Cingulate Cortex:

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,31)=0.467$, $p=0.499$), strain ($F(1,31)=0.002$, $p=0.966$) or fear-conditioning*strain ($F(1,31)=0.578$, $p=0.453$) on the levels of AEA in the ACC of SD and WKY rats (Figure 6.20).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,31)=1.416$, $p=0.243$), strain ($F(1,31)=0.832$, $p=0.368$) or fear-conditioning*strain ($F(1,31)=1.746$, $p=0.196$) on the levels of 2-AG in the ACC of SD and WKY rats (Figure 6.20).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,31)=0.707$, $p=0.407$), strain ($F(1,31)=0.734$, $p=0.398$) or fear-conditioning*strain ($F(1,31)=2.701$, $p=0.110$) on the levels of PEA in the ACC of SD and WKY rats (Figure 6.20).

Two-way ANOVA revealed no significant effect of fear-conditioning ($F(1,31)=0.041$, $p=0.841$), strain ($F(1,31)=0.103$, $p=0.751$) or fear-conditioning*strain ($F(1,31)=1.330$, $p=0.257$) on the levels of OEA in the ACC of SD and WKY rats (Figure 6.20).

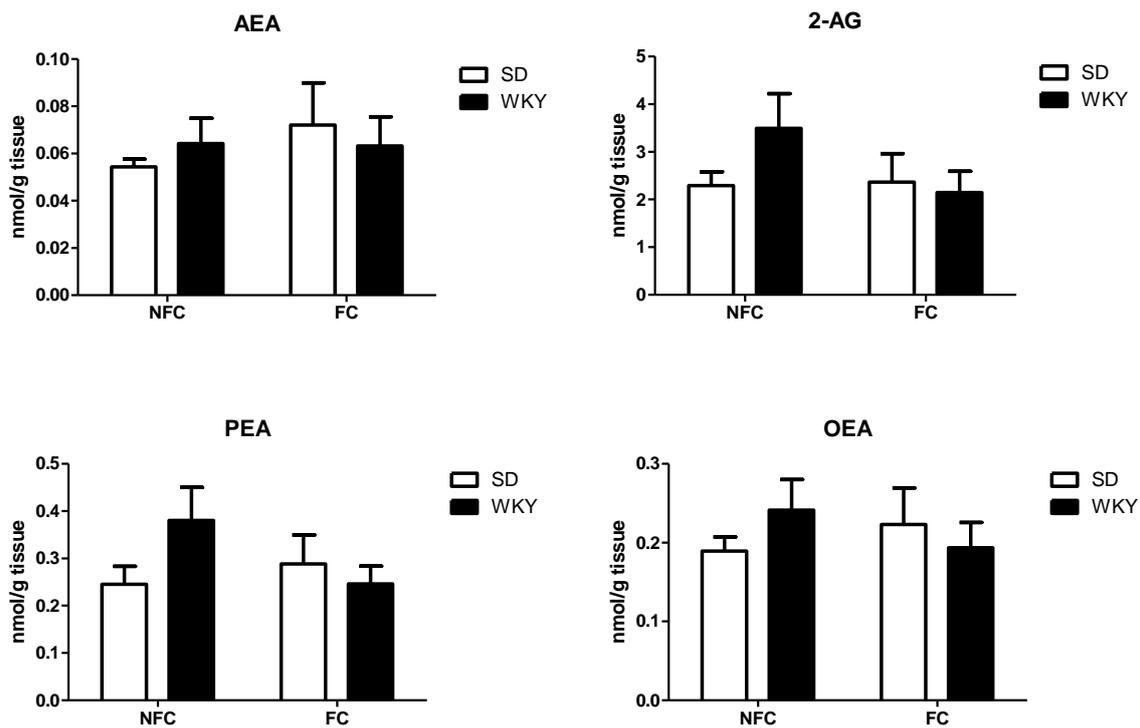


Figure 6.20: Effect of fear-conditioning and strain on AEA, OEA, PEA or 2-AG levels in the ACC of saline-treated rats. All data are expressed as mean \pm SEM ($n=9$ per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned.

6.4 Discussion

The results from the studies described in this chapter demonstrate differences in formalin-evoked nociceptive behaviour, the expression of conditioned fear in the presence and absence of nociceptive tone and FCA between SD and WKY rat strains. Specifically, day 1 fear conditioning revealed that both FC SD and WKY rats exhibited an increased duration in unconditioned aversive behaviours, (time spent freezing or expressing 22kHz ultrasonic vocalisation, duration of escape attempts, duration of startle response to footshock), compared to NFC rats. FC WKY rats spent less time freezing and emitting ultrasonic vocalisation compared to FC SD rats. On test days, SD rats were found to express robust FCA over the course of the 30-minute testing period while WKY rats only expressed FCA for the first 5 minutes of the test trial. Moreover, NFC and FC WKY rats exhibited a higher expression of formalin-evoked nociceptive behaviour compared to SD counterparts. These results support our hypothesis for a dysfunction in the descending inhibitory pain pathway in WKY rats as evidenced by an impaired expression of FCA and increased expression of formalin-evoked nociceptive behaviour. Whether receiving formalin or saline injection, WKY rats expressed little to no fear-related behaviour as indicated by the time spent freezing or emitting 22kHz ultrasonic vocalisation compared to SD rats, indicating another strain-related difference, and possible impairment in the expression of fear-related behaviours. To determine whether strain-related effects were associated with differences in endocannabinoid tone in the mPFC, the levels of AEA, 2-AG, PEA and OEA were measured. Our data indicated a significant strain effect for AEA in the IL of formalin treated rats with trends for lower levels of AEA in WKY rats compared to SD rats. The data also revealed significant strain effects for 2-AG in the IL and PrL of saline treated rats and PEA and OEA in the IL of saline treated rats with trends for increased levels in WKY rats compared to SD rats. These data demonstrate for the first time an impaired expression of FCA and conditioned fear-related behaviours in WKY rats compared to SD rats.

SD rats exhibited a robust reduction in formalin-evoked nociceptive behaviour upon re-exposure to a context previously paired with an aversive stimulus (i.e. footshock), confirming the expression of FCA. As with the other FCA-related studies in this thesis, FCA in SD rats in this study is similar in nature and magnitude to a number of studies, within our own group and others (Fanselow, 1984, Finn et al., 2004, Olango et al., 2012, Rea et al., 2013, Roche et al., 2007, Helmstetter, 1992, Helmstetter and Bellgowan, 1993, Rea et al., 2014a, Rea et al., 2009). In comparison to the first 4 chapters that looked at FCA in Lister-Hooded rats, SD rats

expressed FCA for a similar duration and to a similar magnitude. Unlike SD rats, formalin-treated FC WKY rats only expressed FCA for the first 5 minutes of the testing period. The reduced expression of FCA in WKY rats may in part be explained by the hyperalgesic phenotype displayed by this strain. In the current experiment we showed that NFC WKY rats exhibited enhanced nociceptive behaviour in the formalin model of inflammatory pain when compared to SD counterparts, an effect unchanged in FC WKY rats. This is consistent with previous studies demonstrating that WKY rats display hypersensitivity to noxious stimuli compared with SD rats (Burke et al., 2010, Taylor et al., 2001, O'Mahony et al., 2010, Gunter et al., 2000). Comparing directly to the SD rat strain, previous studies have shown that the WKY rat is hypersensitive to inflammatory pain, exhibiting greater formalin-evoked nociceptive behaviour following intra-plantar injection of formalin into the paw compared to SD rats (Burke et al., 2010, Rea et al., 2014b, Madasu et al., 2016). The results presented here suggest that there may be reduced fear-induced engagement of descending inhibitory pain pathway in WKY rats versus SD rats, accounting for both the hyperalgesic phenotype exhibited by both FC and NFC WKY rats and the impaired expression of FCA in this strain. Further evidence for a reduced engagement of the descending inhibitory pain pathway in WKY vs. SD rats comes from work within our own group. Intra-IPAG administration of ACEA decreased formalin-evoked nociceptive behaviour in SD but not WKY rats, an effect that was associated with decreased c-Fos expression in the dorsal horn of the spinal cord and increased c-Fos in the RVM in SD but not WKY rats, implying a dysfunction of the endocannabinoid system in this top-down control system in WKY rats (Jennings, 2015a).

Fear-conditioning significantly increased the duration of fear-related behaviours (freezing, 22kHz ultrasonic vocalisation and defecation) in SD rats upon re-exposure to the context, irrespective of whether rats received intra-plantar injection of formalin or saline. This finding is in line with previous experiments showing a significant increase in fear-related behaviours upon fear-conditioning (Finn et al., 2004, Olango et al., 2012, Rea et al., 2013, Roche et al., 2007, Rea et al., 2018). Moreover, despite their anxiogenic phenotype, FC WKY rats expressed little or no freezing and 22kHz ultrasonic vocalisation irrespective of intra-plantar formalin/saline injection. FC WKY rats (both saline and formalin treated) only freeze for the first 5 minutes of the testing period and do not exhibit an increase in 22kHz ultrasonic vocalisation. Moreover, both behaviours are expressed to a lower degree by WKY rats when compared to SD rats. Defecation is also a measure of fear/emotionality in this fear-conditioning paradigm. Regardless of treatment (formalin/saline) FC SD rats expressed a higher number of

faecal boli compared to WKY rats. These results imply that despite exhibiting an anxiety-depressive phenotype, in the present study WKY rats have an impaired expression of conditioned fear-related behaviours. While this impaired expression of fear-related behaviours may be due to the hyperalgesic phenotype of the WKY rat strain this is probably not the cause as fear-related behaviours were lower in both formalin- and saline-treated FC WKY rats compared with FC SD counterparts.

It is possible that WKY rats have impaired learning or memory and are not able to make the association between the US and CS as well as SD rats, or that they are unable to consolidate or recall the aversive memory of receiving footshock in the conditioning arena. In contrast to the results presented in this chapter, following a cued fear-conditioning paradigm WKY rats displayed conditioned fear responses and increased freezing behaviour in a comparable manner to SD rats. In this study, WKY rats displayed conditioned fear-responses both 15 minutes and 24 hours post-conditioning (Ledoux et al., 1983). Similarly, WKY maintained an increased fear-response following cued fear-conditioning 14 days after the initial conditioning phase, an effect not seen in Wistar rats (DaSilva et al., 2011). Shock stress without the CS had no effect on freezing behaviour in WKY rats indicating the importance of both the CS and US and the ability of WKY to associate the two and exert a conditioned fear response (DaSilva et al., 2011). These findings imply that the results seen in this chapter are probably not due to an impairment in learning and memory. However, the study presented in this chapter uses contextual fear-conditioning while the aforementioned studies use cued fear-conditioning and so WKY rats may have an impairment in the ability to associate contextual vs cue conditioning. In comparison, studies have indicated no learning/memory-related deficit in WKY rats in the Morris water maze and novel object recognition tasks in comparison to SD and Wistar rats respectively (Ferguson and Cada, 2004, Langen and Dost, 2011). It is possible that the impairment in fear-related behaviours may be due to a lack of reactivity of the WKY rat due to a depressive-like state (Pare and Redei, 1993). Compared to SD rats, the WKY rat displays a high anxiety-like phenotype in the open field and elevated plus maze tests, acoustic startle response test and a depressive-like phenotype in the forced swim test (Drolet et al., 2002, Ferguson and Cada, 2003, Nosek et al., 2008, Glowa and Hansen, 1994, McAuley et al., 2009, Servatius et al., 1998, Malkesman and Weller, 2009, Pare, 1992, Pare and Redei, 1993). Jennings et al., however, found that repeated forced swim stress enhanced formalin-evoked nociceptive behaviour in SD rats but reduced it in WKY rats, despite the anxiogenic and hyperalgesic phenotype of the WKY rat strain (Jennings et al., 2015). Thus, the type of stressor

used may influence the expression of pain and fear in the WKY rat strain. Our work is the first to compare formalin- and saline-treated SD and WKY rats in this fear-conditioning paradigm.

It is possible that the difference in the expression of FCA and conditioned fear responding may be due to the WKY rats reactivity to footshock stimulus and conditioning paradigms on day 1. To address this, I measured and scored unconditioned aversive behaviour and locomotor activity in both strains on the conditioning day. During fear-conditioning the distance moved and duration of walking were decreased in SD and WKY rats, compared with non-fear-conditioned counterparts. The duration of both rearing and grooming was decreased in FC SD but not WKY rats. NFC WKY rats exhibited a lower duration of rearing and a trend for decreased duration of grooming compared to SD rats. These results may be due to the hypolocomotor phenotype of the WKY rat associated with the anxiodepressive phenotype of this strain (Burke et al., 2010, Olango et al., 2012, Jennings 2015a). Indeed, antidepressants have been shown to decrease hypolocomotion in the WKY rat in the forced swim test of depression (Browne et al., 2015, Carr et al., 2010, Carr and Lucki, 2010). The rats receiving the footshock on conditioning days express more freezing and 22kHz ultrasonic vocalisation compared to NFC counterparts and attempt to escape the arena more. WKY rats express less of these unconditioned aversive behaviours than SD rats during footshock exposure, implying the footshock is having less of an effect in WKY rats. However, both SD and WKY rats have an equally increased response to footshock compared to rats not receiving footshock. It is possible that the WKY rats are anxious to begin with and due to this high anxiety-like phenotype are just not reacting as much as SD rats in this fear-conditioning paradigm or that the WKY rats manifest their anxiety in a different manner to SD rats. WKY rats tend to exhibit behavioural inhibition in paradigms involving stressful stimuli, in particular the open field test (Drolet et al., 2002, Ferguson and Cada, 2003, Nosek et al., 2008). In comparison, shock reactivity during the conditioning phase of a cued fear-conditioning paradigm was the same between WKY, Brown Norway and Lewis Evans rats (Schaap et al., 2013). During this conditioning session WKY rats showing showed a learning curve with the duration of freezing behaviour increasing over the CS-US pairings. During the test phase of this paradigm (24 hours later) WKY rats, along with all other strains, displayed a markedly increased duration of freezing during the first CS bin, compared to 30 seconds before the CS onset, suggesting that the CS-US association was present in WKY rats (Schaap et al., 2013). The lack of reactivity may be due to the WKY rat's depressive phenotype. One way to determine stress levels would be to measure plasma levels of stress hormones such as cortisol. It is known that WKY rats

also exhibit enhanced stress hormone and HPA axis responses to stress when compared to control comparator rat strains (Rittenhouse et al., 2002, Solberg et al., 2004). With regards to the paradigm tested here, measuring cortisol levels before and after conditioning and test days in both SD and WKY rats would provide insight into HPA axis reactivity throughout the testing process and allow us to draw comparisons between the two strains. Though not carried out in the present chapter, future studies should measure the levels of cortisol in these rats to determine the baseline stress levels during this fear-conditioning paradigm.

On our test days (day 2), formalin-treated, NFC WKY rats exhibited a decreased distance moved and decreased duration of walking, rearing and grooming compared to SD rats, an effect not seen in FC counterparts. These results suggest that in the absence of fear-conditioning, WKY rats are naturally more anxious, displaying less locomotor and exploratory behaviour compared to SD rats. This is in line with other studies showing hypolocomotion in WKY rats compared to SD rats in the open field and formalin test (Burke et al., 2010, Olango et al., 2012, Jennings, 2015a). In saline-treated rats, FC SD rats exhibit decreased distance moved and walking compared to NFC SD and FC WKY rats. FC SD rats freeze more than NFC SD and FC WKY, and this be related to the decrease in distance moved and walking seen in this group. NFC WKY rats rear less and the NFC SD rats, an effect also seen in formalin treated rats and may further indicate a naturally anxiogenic phenotype in this strain and thus less exploratory behaviour such as decreased rearing.

To determine if the strain related differences in this study were associated with alterations in the endocannabinoid system in the mPFC we measured the levels of endocannabinoids and N-acylethanolamines in the mPFC via mass spectrometry. The results suggest that there are no significant differences in AEA, 2-AG, PEA or OEA in formalin- or saline-treated rats but there were some interesting significant strain effects. In formalin-treated rats there was a significant strain effect for AEA in the IL with lower levels of AEA in WKY rats compared with SD counterparts. It is possible that the reduced levels of AEA in the IL in this strain may account for the hyperalgesic phenotype in this rat strain as these animals were all administered formalin, a hypothesis that will be tested in Chapter 7. Immunoblot analysis has previously revealed increased FAAH expression in the frontal cortex of this rat strain when compared to SD counterparts (Vinod et al., 2012). FAAH is the main degrading enzyme for AEA and so this may account for the decreased levels of AEA in WKY rats in our study. In saline-treated rats there was a significant strain effect in the IL for 2-AG, OEA and PEA with increased levels of these endocannabinoids in the WKY rats compared with SD rats. There was also a significant

strain effect in the PrL for 2-AG in saline-treated rats with increased levels of 2-AG in WKY vs SD rats. It is possible that the increased levels of these neuromodulators in saline-treated rats may account for the reduction fear-related behaviours. As stated in chapter 5, the levels of 2-AG in the brain tend to increase in rats exposed to fearful/stressful stimuli (Bluett et al., 2017, Morena et al., 2015, Patel et al., 2005). It is possible that in the absence of formalin these neurotransmitters are released to aid in the extinction of fear-related behaviours and this is why in WKY rats we see the expression of fear-related behaviour for less time compared to SD rats. Further drug-related studies targeting these cannabinoids in the mPFC may reveal the mechanism underlying the hyperalgesic phenotype and impaired expression of fear-related behaviours in WKY rats.

In conclusion, these results demonstrate for the first time a strain-related difference in pain suppression upon re-exposure to a context previously paired with an aversive stimulus (FCA) and imply a dysfunction in the descending inhibitory pain pathway of WKY rats. Moreover, these results highlight the hyperalgesic phenotype in WKY rats along with reduced expression of fear-related behaviours. It is possible that deficits in AEA in the IL may play a role in the hyperalgesic phenotype of WKY and increased levels of 2-AG, OEA and PEA in the IL and 2-AG in the PrL may account for the reduced fear-related behaviour in the WKY rats but further studies would be necessary to test these hypotheses. Future studies should investigate pharmacological modulation of the endocannabinoid system of SD and WKY in the IL and PrL and examine their effects on formalin-evoked nociceptive behaviour in the absence and presence of conditioned fear. Overall these results highlight the importance of genetic background in pain, endogenous analgesia and fear responding.

Chapter 7: Investigating the effects of pharmacological modulation of the endocannabinoids in the rat infralimbic cortex (IL) on formalin-evoked nociceptive behaviour in WKY versus SD rats.

7.1 Introduction

Both preclinical and clinical studies have highlighted an important interaction between stress/anxiety/fear and pain. The context, nature, duration and intensity of a stressor all have profound influences on stress-induced modulation of pain. In general, positive emotions tend to inhibit pain while negative emotions with low arousal augment pain and negative emotions with high arousal inhibit pain (de Wied and Verbaten, 2001, Dougher, 1979, Meagher et al., 2001, Rhudy and Meagher, 2000, Rhudy and Meagher, 2001, Rhudy and Meagher, 2003a, Rhudy and Meagher, 2003b). Clinically, neuropsychiatric disorders such as anxiety and depressive disorders are often accompanied by chronic pain complaints and *vice versa* (Asmundson and Katz, 2009). This co-morbidity is often more disabling to patients than either disorder alone, making the treatment more costly. Understanding the mechanisms that underlie the pathophysiology of these co-morbid disorders could lead to the potential discovery of effective new therapeutics targets and drugs.

The WKY rat has been used to study the influence of negative affect on pain, as well as the role genetic background plays. As explained in the previous chapter, the WKY rat is an inbred rat strain that exhibits a stress hyper-responsive and anxiodepressive phenotype compared to SD rats (Burke et al., 2010). The WKY rat also exhibits a hyperalgesic phenotype upon exposure to inflammatory, mechanical or visceral pain (Burke et al., 2010, Taylor et al., 2001, Wang et al., 2012), and reduced expression of FCA as demonstrated in Chapter 6 of this thesis. The mechanisms underpinning the hyperalgesia in this rat strain are still poorly understood.

The endocannabinoid system is a key neuromodulatory system, involved in several physiological processes including pain and anxiety. These have been discussed in detail throughout this thesis. Evidence is now emerging to suggest that the endocannabinoid system in WKY rats is different to that in comparator rat strains. The expression of the gene encoding CB₁ receptors in the locus coeruleus of WKY rats is significantly higher than in SD rats (Pearson et al., 2006). Similarly, CB₁ receptor expression is significantly higher and AEA

levels are lower in the frontal cortex and hippocampus of WKY rats compared with Wistar rats (Vinod et al., 2012). Moreover, chronic systemic administration of the FAAH inhibitor URB597 had an antidepressant-like effect in WKY rats, suggesting that the endocannabinoid system may play a key role in the genetic predisposition of the WKY rat to a depressive-like phenotype (Vinod et al., 2012). Previous work in our laboratory has suggested that endocannabinoid-CB₁ receptor signalling in the RVM is reduced in WKY rats, thereby contributing to their hyperalgesic response to intra-plantar injection of formalin (Rea et al., 2014b). Repeated forced swim stress can reduce formalin-evoked nociceptive behaviour in WKY rats compared to SD rats, an effect that may be mediated by altered endocannabinoid system expression in the spinal cord and amygdala (Jennings et al., 2015). In addition, PPAR γ expression in the IPAG is higher in saline-treated WKY rats while PEA and OEA levels were higher in formalin-treated WKY rats. PPAR γ antagonism in the IPAG increases formalin-evoked nociceptive behaviour in WKY, but not SD, rats (Okine et al., 2017). It is possible that differences in PPAR signalling in the PAG of these rat strains may account for their differential response to pain. Similarly, TRPV1 expression is significantly higher in the IPAG and lower in the dIPAG of formalin-treated WKY rats compared to SD rats. Depending on the sub column of the PAG, TRPV1 receptor activation or inhibition differentially altered formalin-evoked nociceptive behaviour (enhanced or attenuated) in SD rats compared to WKY rats, suggesting a role for TRPV1 in the PAG in the hyper-responsivity of the WKY rat to pain (Madasu et al., 2016). To date, the role of the endocannabinoid system in the IL of WKY rats in comparison to SD rats has not yet been evaluated.

The mPFC has a high expression of CB₁ receptors (Herkenham et al., 1991, Tsou et al., 1998a). The mPFC is subdivided into three subregions, the IL, PrL and ACC. The IL is an important region for its key roles in pain, fear and FCA. It may also function in the modulation of top down responses to pain and fear. The results presented in Chapter 6 suggest a functional impairment in the descending inhibitory pain pathway of WKY rats as evidenced by the reduced expression of FCA in WKY rats versus SD counterparts. Mass spectrometry revealed a significant overall strain effect in the levels of AEA in the IL of formalin treated WKY rats with lower levels in this strain compared with SD counterparts (see Figure 7.1). Thus, I hypothesised that lower levels of AEA in the IL of the WKY rat may account for its hyperalgesic phenotype.

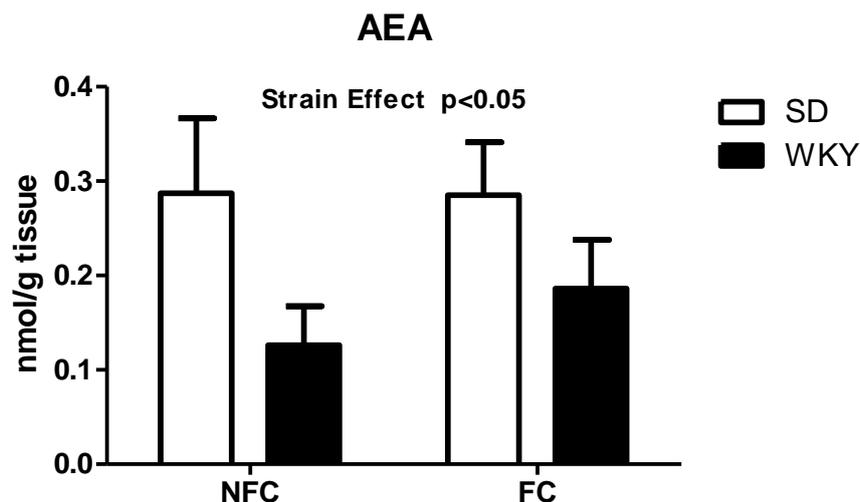


Figure 7.1: Effect of fear-conditioning on levels of AEA in the IL of formalin-treated SD and WKY rats. All data are expressed as mean \pm SEM ($n=6-9$ per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; NFC, Non-fear-conditioned; FC, fear-conditioned.

Therefore, the overall aims of the study described in this chapter were:

- To confirm increased formalin-evoked nociceptive behaviour in naïve WKY rats versus SD rats
- To investigate the effects of intra-IL microinjection of the FAAH inhibitor URB597, the AEA analog methanandamide (mAEA), the CB₁ receptor agonist ACEA or the CB₁ receptor antagonist AM251 on formalin-evoked nociceptive behaviour in WKY versus SD rats
- To measure CB₁ receptor expression in the IL, PrL and ACC of formalin-injected SD and WKY rats.

7.2 Materials and Methods

7.2.1 Animals:

Male Sprague-Dawley (269 – 335g Harlan Italy) and Wister-Kyoto rats (239 – 262g; Harlan Laboratories, Belton, Loughborough, UK) were used in this study. Before surgery animals were housed 3 per flat bottomed cage (45 x 20 x 20cm) containing wood shavings as bedding (LBS, Surrey, UK). Animals were kept at a constant temperature ($21^{\circ}\text{C} \pm 2^{\circ}\text{C}$) under standard lighting conditions (12:12h light–dark, lights on from 0800 to 2000h). Experiments were carried out during the light phase between 0800 and 1700h. Food (Harlan-Teklad-2014 Maintenance Diet, Harlan Laboratories, Belton, Loughborough, UK) and water were provided *ad libitum*. The experimental protocol was carried out after approval by the Animal Care and Research Ethics Committee, National University of Ireland, Galway, under license from the Health Products Regulatory Authority and in compliance with the European Communities Council directive 86/609 and 2010/63 and conformed to the ARRIVE guidelines.

7.2.2 Cannulae implantation:

See Chapter 2 section 2.2.2 for details on cannula implantation via stereotaxic surgery.

Stainless steel guide cannulae (9mm length, 22G, Plastics One Inc., Roanoke, VA) were stereotaxically implanted 1mm above the left and right IL (SD coordinates: AP = +2.3mm from Bregma, RML = -1.5mm, LML = +1.5mm at an angle of 12° , DV: -3.7mm; WKY coordinate: AP = +2.15mm from Bregma, RML = -1.5mm, LML = +1.5mm at an angle of 12° , DV: -3.7mm) from the meningeal dura matter according to the rat brain axis published by Paxinos and Watson, 1998 (Paxinos and Watson, 1998).

Following surgery, animals were singly housed and a single daily dose of enrofloxacin (2.5 mg/kg, *s.c.*, Baytril; Bayer Ltd., Dublin, Ireland) was administered for the following four days. A minimum of 6 days was allotted to allow rats to recover before any experimentation commenced. During this period, the rats were handled, stylets checked, and their body weight and general health monitored.

7.2.3 Chemicals and drug preparation:

The anandamide analog methanandamide (mAEA) [(R)-N-(2-Hydroxy-1-methylethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide) Cambridge, UK] and the fatty acid amide hydrolase (FAAH) inhibitor and indirect agonist URB597 [(30-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate) Sigma-Aldrich, Dublin, Ireland] were prepared from a 5mg stock to a working concentration of 0.1µg/0.3µl DMSO (dimethylsulfoxide, 100%; Sigma-Aldrich, Dublin, Ireland) and from a 0.2mM stock to a working concentration of 0.1mM/0.3µl DMSO, respectively.

The selective CB₁ receptor agonist Arachidonyl-2'-chloroethylamide (ACEA) [*N*-(2-Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide, Tocris, UK] and the CB₁ receptor antagonist AM251 [*N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide, Tocris UK] were prepared from a 5mg stock to a working concentration of 2mM/0.3µl DMSO and from a 5mg stock to a working concentration of 0.05pmol/0.3µl DMSO respectively.

These choice of these doses of URB597, mAEA, ACEA and AM251 was based on previous work carried out by our laboratory and evidence from the literature which indicated *in vivo* efficiency in rat models of pain and fear (Rubino et al., 2008b, Lisboa et al., 2010, Ford et al., 2011, Jennings, 2015b, Moreira et al., 2007, Rea et al., 2018).

A solution of 2.5% formalin (Sigma-Aldrich, Dublin, Ireland) was prepared from a 37% stock solution diluted with 0.9% sterile saline.

7.2.4 Experimental procedure:

On the test day, rats received bilateral microinjection of either mAEA, URB597, ACEA, AM251 or vehicle (100% DMSO), see Table 7.1 for n numbers. An injector was inserted through the guide cannulae into the IL protruding 1mm beyond the tip of the guide cannula. A volume of 0.3µl was injected into the IL over the course of 60s via both guide cannulae using a 1µl Hamilton micro syringe attached to polyethylene tubing and a Harvard PHD2000 infusion pump (Harvard Apparatus, Kent, UK). The injector was left in the guide cannula for 60s before removal to allow for complete drug infusion. Animals were immediately placed in a darkened Perspex arena (30 x 30 x 40cm; 30 lux) for a 10-minute pre-formalin period where general locomotor exploratory behaviours, walking, rearing, grooming and distance moved were recorded and rated. Animals were then removed from the arena where they received intra-

plantar injection of 50µl formalin (2.5% in 0.89% saline, Sigma, Ireland) into the right hind-paw under brief isoflurane anaesthesia (2% in O₂; 0.5L/min). Rats were placed back into the Perspex arena, the test began when the rat righted itself, and the trial lasted 60 minutes after which rats were removed and euthanized by decapitation. The brains and spinal cords were harvested for post-mortem analysis. The diameter of the right hind-paw was measured using Vernier callipers immediately prior to and 60 minutes post intra-plantar formalin injection as an index of formalin-induced oedema. The number of pellets post-formalin period were also counted.

This design resulted in 6 experimental groups per experiment (Starting n=12-24 per group for surgery; final n=8-21 per group for data analysis) as illustrated in Table 7.1. Animal behaviour was recorded over the 10-minute pre-formalin trial period and 60-minute formalin trial period using a video camera located beneath the observation chamber with video feed from this camera recorded for behavioural analysis.

After 60 minutes in the arena for the test trial, rats were removed and euthanised by decapitation. Brains were rapidly removed within a 2-minute timeframe, snap-frozen on dry ice and stored at -80°C.

Strain	Drug Treatment				
	VEH	mAEA	URB597	ACEA	AM251
SD	17	8	9	11	10
WKY	21	9	10	8	11

Table 7.1: Summary of experimental groups. (SD: Sprague Dawley; WKY: Wistar-Kyoto; VEH: Vehicle; mAEA: methanandamide)

7.2.5 Brain removal:

See chapter 2 section 2.2.5.

7.2.6 Behavioural analysis:

Ethovision XT 7.0 software package (Noldus, Wageningen, The Netherlands) was used to analyse behaviour, allowing for continuous event recording over each 60 minute trial test trial

and 10-minute pre-formalin trial. Formalin-evoked nociceptive behaviour was scored according to the weighted composite pain scoring (CPS) technique described in chapter 2. Exploratory behaviour during the pre and post-formalin period, distance moved, walking, rearing and grooming were also assessed.

7.2.7 Histological verification of intracerebral injection sites:

See chapter 2 section 2.2.7.

7.2.8 Tissue isolation by Palkovits punch:

See chapter 3 section 3.2.8.

For VEH treated rats, half of the punches were reserved for analysis with mass spectrometry while the other half were reserved for analysis using western immunoblotting.

7.2.9 Measurement of levels of endocannabinoids in the medial prefrontal cortex:

See chapter 2 section 2.2.9 with the same amendments as chapter 3 section 3.3.9.

7.2.10 Western immunoblotting:

Frozen mPFC punches (IL: 1.45 ± 0.2 mg; PrL 1.8 ± 0.25 mg; ACC: 2 ± 0.3 mg) were lysed by brief 3s sonication in 25 μ l radio-immunoprecipitation assay (RIPA) lysis buffer (150mmol/L NaCl, 25mmol/L Tris-HCl, pH 7.6, 0.5% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 1mmol/L Na₃VO₄, 10mmol/L NaF containing 1% protease inhibitor cocktail [Sigma-Aldrich, Ireland]) at a ratio of 1:10 (w/v) in a 1.5ml microcentrifuge tube. Following homogenisation the microcentrifuge tube was placed on a shaker for 45 minutes at 4°C with gentle agitation to allow for complete dissociation of nucleo-protein complexes and then centrifuged at 14000g (Eppendorf Centrifuge 5415R Stevenage, UK) for 20mins at 4°C to separate the precipitate and the supernatant. The supernatant was collected, and protein content determined by Bradford assay. The Bradford assay consisted of adding 250 μ l Bradford reagent (Sigma-Aldrich, Ireland) to 5 μ l of unknown samples or standards (in triplicate) on a 96-well plate. After a 5-minute incubation time, absorption at 570 nm wavelength was determined. Protein concentrations of the samples were determined using an 8 point standard

curve constructed using bovine serum albumin (BSA) standards (0-2mg/ml). The samples were equalised to 1mg/ml after determining the protein concentration. 24µg (8µl of sample loading buffer is added to 24µl of protein sample) of protein sample in loading buffer (4X sample loading buffer: 25% v/v 1 mol/L Tris HCl, pH 6.8, 5% w/v sodium dodecyl sulfate[SDS], 20% v/v glycerol, 2.5% Bromophenol blue [0.2% w/v in 100% ethanol], 7M Urea, and 20% v/v of 2-mercaptoethanol, made up to a total volume of 20mL in distilled water) was boiled at 100°C for 5 minutes, briefly centrifuged, and subjected to 9% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) at a constant voltage of 120mV for 2hrs. To directly visualize protein bands a pre-stained protein ladder (Chameleon Duo Pre-stained Protein Ladder [P/N 928-60000]; LI-COR Biosciences Abingdon Park, Oxford, UK) was also loaded on the gel (5µl). The separated protein samples were electroblotted onto a nitrocellulose membrane (Nitrocellulose membrane, CAS# 9004-70-0; Bio-Rad, Ireland) at 100mV for 45min. Protein transfer efficiency was verified by ponceau dye (0.1% ponceau dye in 5% acetic acid; Sigma-Aldrich, Ireland) staining. The blots were placed in a small plastic container that was then filled with ponceau dye and rocked back and forth for about 1 minute. The success of the transfer is confirmed by the instantaneous appearance of numerous protein bands on the membrane and the absence of any bubble patches on the membrane. Membranes were blocked in 5% non-fat dry milk in 0.05% Tris-buffered saline/Tween 20 (TBST – see appendix for recipe) solution for 1hr at room temperature and incubated with rabbit polyclonal antibody to the CB₁ receptor (C-term) (1:200, catalogue no. 10006590; Cayman Chemical, MI) and mouse monoclonal antibody to β-Actin (1:10,000, A5441; Sigma-Aldrich, Ireland) diluted in 5% milk/0.05% TBST for 3hrs at room temperature and then overnight at 4°C. 24hr after start of primary antibody incubation, the membrane was washed in washing buffer (0.1% TBST) in four 5-minute washes. Next, membranes were incubated in secondary antibody solution containing IRDye conjugated goat anti-rabbit (k800) and goat anti-mouse (k700) (LI-COR Biosciences Abingdon Park, Oxford, UK) diluted 1:10,000 in 1% milk/0.1% TBST for 1hr at room temperature. Four 5-minute washing steps were then performed with washing buffer (0.1% TBST) and one final 5-minute wash in distilled H₂O. Blots were scanned on a LI-COR Odyssey imager. IR band intensities for CB₁ receptor protein expression (~63-kDa) and β-actin (~42-kDa) for each sample were generated automatically using the background subtraction method of the LI-COR Image Studio Ver. 2.0 imaging software. The ratio of CB₁ receptor intensity to β-actin intensity was then calculated for each sample. The intensity ratio of each sample was then expressed as a percentage of the average of intensity ratio for the SD-VEH group. Full details of the composition of all buffers/solutions used are provided in Appendix 1.

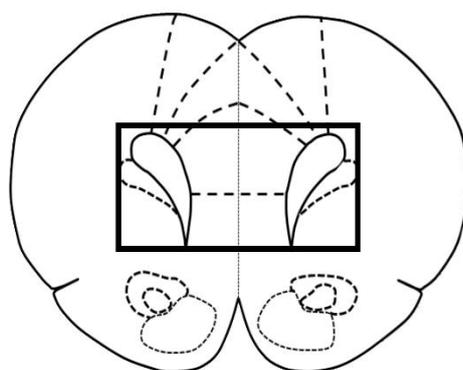
7.2.11 Statistical analysis:

The IBM SPSS statistical software package (SPSS v23.0 for Microsoft Windows; Chicago, IL, USA) was used to analyse all data. Normality and homogeneity of variance were assessed using Shapiro-Wilk's and Levene's test, respectively. Behavioural data and mass spectrometry data were analysed by 2-way ANOVA, with strain and drug treatment as factors. For western immunoblotting an independent samples, two-tailed t-test was run. Time course behavioural data were analysed by 2-way repeated measures ANOVA with time as the within-subjects factor and strain and drug treatment as the between- subjects factors. Sphericity was tested using Mauchly's Test for Sphericity. If sphericity was violated a Greenhouse-Geisser correction was used. Post-hoc pairwise comparisons were made with Tukey's test when appropriate. Defecation (pellet number) data were non-parametric and analysed using Kruskal-Wallis test followed by Dunn-Bonferroni post-hoc where appropriate. Data were considered significant when $p < 0.05$. Results are expressed as group means \pm SEM.

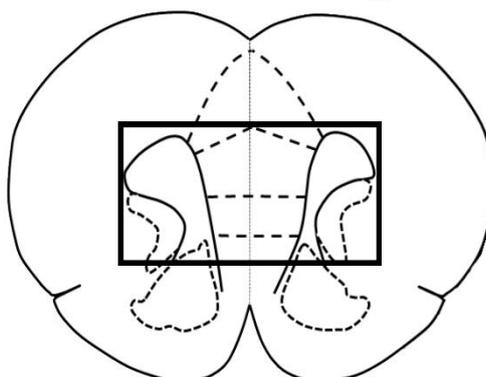
7.3 Results

7.3.1 Histological verification of injector site location

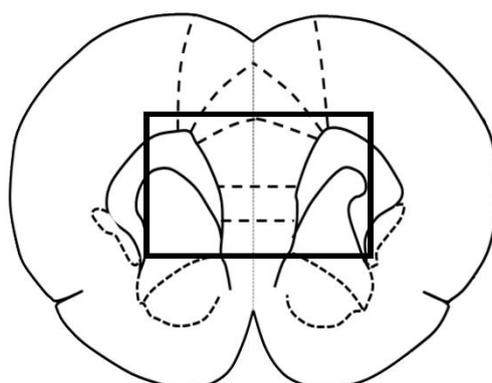
79% of the injections were placed within the borders of the IL for SD and WKY rats (Figure 7.2) with the remaining 21% positioned outside the IL in either the corpus callosum or PrL. Only the results of experiments in which bilateral injections were correctly positioned in both the right and left IL were included in the analysis.



+3.2 mm from Bregma



+2.7 mm from Bregma



+2.2 mm from Bregma

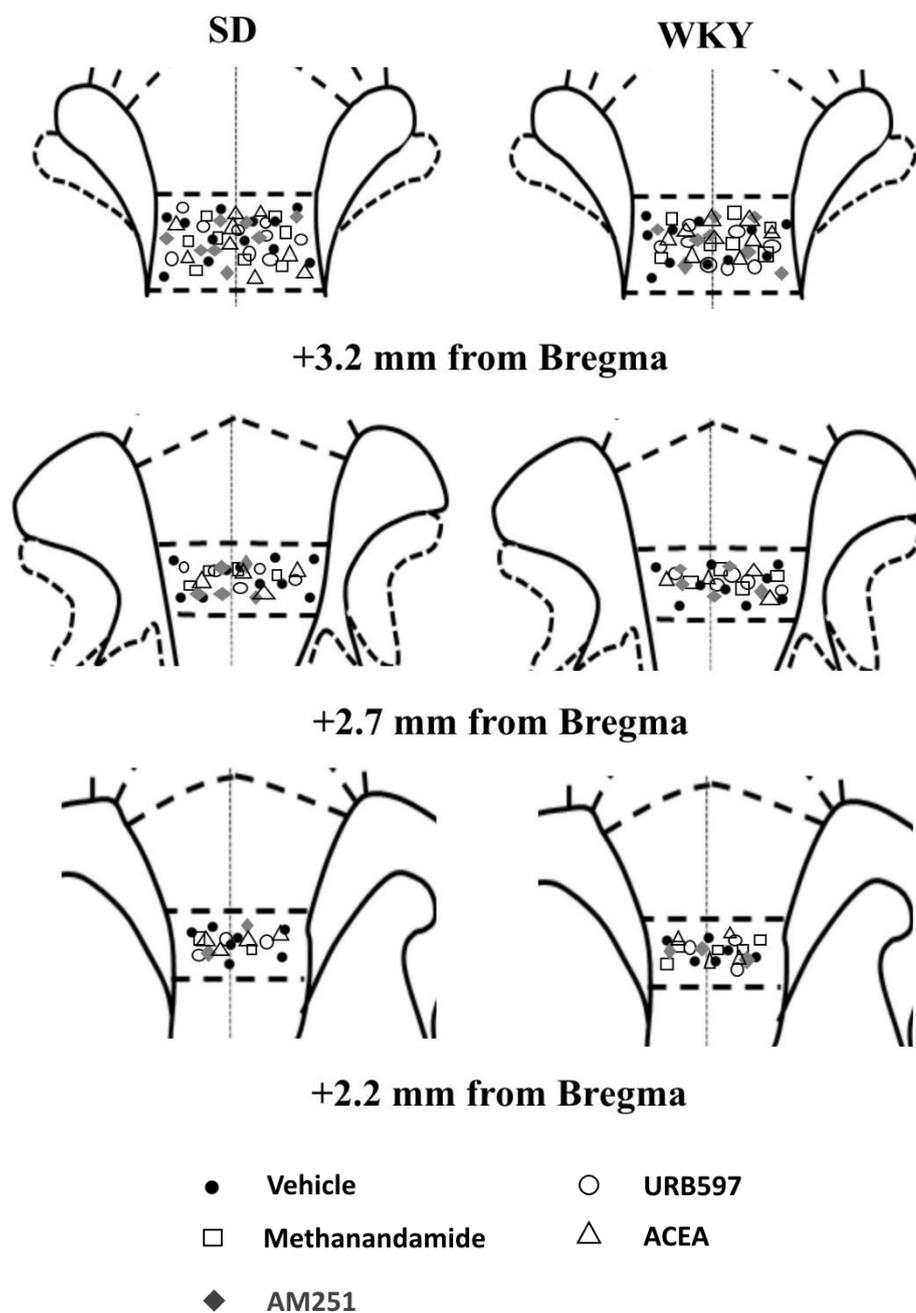


Figure 7.2: Histological verification of injector site location. There are fewer sites than numbers as some injection sites overlap. SD: Sprague-Dawley, WKY: Wistar-Kyoto.

7.3.2 Effects of intra-IL administration of a CB₁ receptor agonist, antagonist, FAAH inhibitor or AEA analog, on pre-formalin general locomotor and exploratory behaviour

Directly after microinjection, rats were placed in the formalin arena and general exploratory behaviours were recorded over a 10-minute period, prior to intra-plantar formalin injection (Table 7.2).

Two-way ANOVA revealed a significant main effect of strain ($F(1,104)=33.204$, $p<0.001$), treatment ($F(4,104)=2.949$, $p<0.05$) and strain*treatment ($F(4,104)=4.810$, $p<0.001$) on the distance moved over the course of the 10-minute pre-formalin testing period. Further post-hoc analysis revealed that distance moved in VEH-treated WKY rats was significantly lower than SD counterparts (SD VEH vs WKY VEH, $p<0.05$), this remained unchanged upon treatment with ACEA, AM251, URB597 and mAEA (Table 7.2).

Two-way ANOVA revealed a significant main effect of treatment on walking ($F(4,104)=2.521$, $p<0.05$) but not strain ($F(1,104)=0.085$, $p=0.772$) or strain*treatment ($F(4,104)=1.117$, $p=0.353$). Further post-hoc analysis revealed no significant pairwise, between-group differences (Table 7.2).

Two-way ANOVA revealed a significant effect of strain ($F(1,104)=10.345$, $p<0.01$) and strain*treatment ($F(4,104)=3.021$, $p<0.05$) but not treatment alone on the duration of rearing ($F(4,104)=0.711$, $p=0.586$). Further post-hoc analysis revealed no significant pairwise, between-group differences (Table 7.2).

Two-way ANOVA revealed a significant effect of strain ($F(1,104)=6.259$, $p<0.05$) on the amount of time spent grooming but not treatment ($F(4,104)=1.431$, $p=0.229$) or strain*treatment ($F(4,104)=2.069$, $p=0.09$). Post-hoc analysis revealed no significant pairwise, between-group differences (Table 7.2).

Treatment Group	Distance Moved (cm)	Walking (s)	Rearing (s)	Grooming (s)
SD-VEH	1722±147	108±6	101±9	19±2
SD-URB597	2013±62	130±6	100±20	23±5
SD-mAEA	1946±206	111±6	99±19	31±16
SD-ACEA	2328±85	95±8	67±11±	33±8
SD-AM251	2344±102	102±10	82±10	31±4
WKY-VEH	1268±45*	107±6	65±7	13±2
WKY-URB597	1188±57*	125±12	46±6	30±7
WKY-mAEA	1253±56*	95±7	39±2	34±18
WKY-ACEA	1320±59*	106±7	85±11	21±9
WKY-AM251	1277±78*	122±14	81±12	13±3

Table 7.2: Effects of intra-IL administration of a CB₁ receptor agonist, antagonist, FAAH inhibitor or AEA analog on general locomotor activity and exploratory behaviour in SD and WKY rats prior to formalin injection during the 10-minute period post intra-IL microinjection. *p<0.05 vs SD counterpart; #p<0.05 vs. SD VEH (Tukey's). Data are presented as mean ± SEM (n=8-21 per group). (SD: Sprague-Dawley, WKY: Wistar-Kyoto, VEH: vehicle).

7.3.3 Effects of intra-IL administration of a CB₁ receptor agonist, antagonist, FAAH inhibitor or AEA analog on formalin-evoked nociceptive behaviour in SD and WKY rats

Intra-plantar injection of formalin produced a robust nociceptive response in the form of elevation, licking, biting and shaking of the right hind-paw in both SD and WKY rats. Two-way ANOVA revealed no significant effect of strain ($F(1,104)=0.234$, $p=0.629$), treatment ($F(4,104)=1.800$, $p=0.134$) or strain*treatment ($F(4,104)=2.003$, $p=0.099$) on the change in hind paw diameter pre- versus post-formalin injection (Figure 7.3).

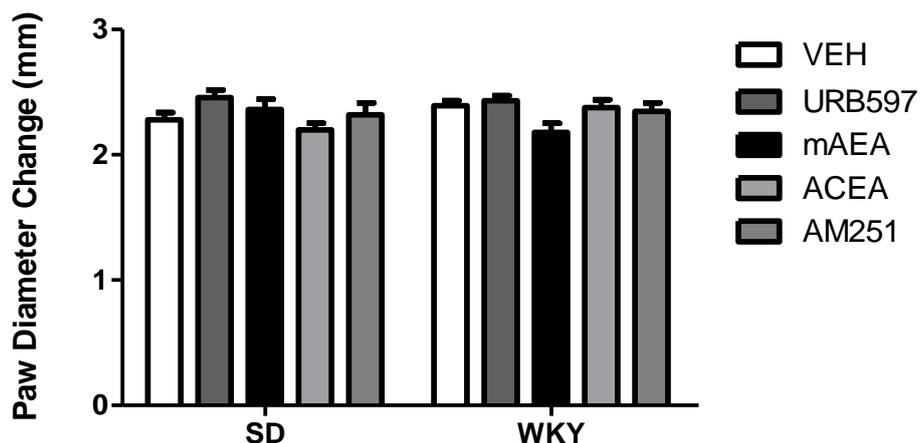


Figure 7.3: Effects of intra-IL administration of a CB₁ receptor agonist, antagonist, FAAH inhibitor or AEA analog on paw diameter change (mm) pre and post formalin injection (oedema) in SD and WKY rats. All data are expressed as mean \pm SEM (n=8-21 per group). VEH: Vehicle; SD: Sprague-Dawley; WKY: Wistar-Kyoto.

Two-way ANOVA revealed a significant main effect of strain ($F(1,104)=22.687$, $p<0.001$), treatment ($F(4,104)=7.613$, $p<0.001$) and strain*treatment ($F(4,104)=4.234$, $p<0.01$) on CPS over the entire 60-minute testing period. Further post-hoc analysis revealed a significant reduction in CPS in SD mAEA and URB597 treated rats compared to SD VEH treated rats, an effect not seen in WKY rats (SD VEH vs. SD mAEA, $p<0.001$; SD VEH vs. SD URB597, $p<0.01$). mAEA and URB597 treated SD rats exhibited significantly lower formalin-evoked nociceptive behaviour compared to WKY-treated counterparts (SD mAEA vs WKY mAEA, $p<0.01$; SD URB597 vs. WKY URB597, $p<0.05$), an effect not seen in VEH-treated SD and WKY rats (SD VEH vs. WKY VEH, $p=1.000$) (Figure 7.4).

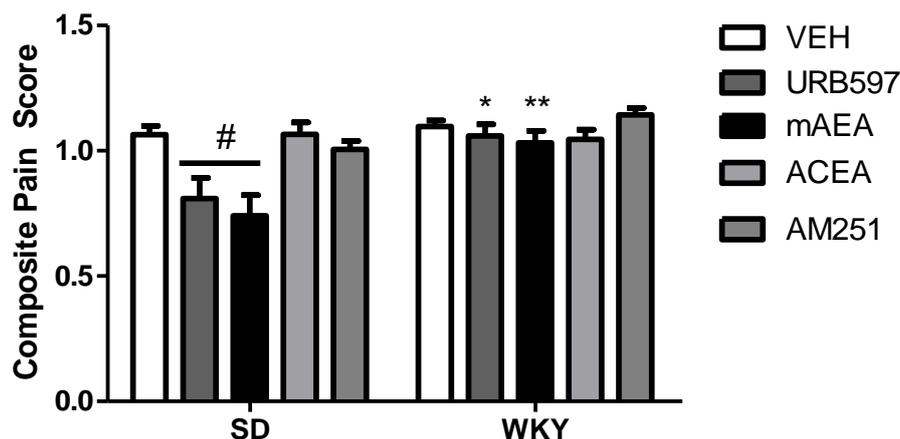


Figure 7.4: Effects of intra-IL administration of a CB₁ receptor agonist, antagonist, FAAH inhibitor or AEA analog on formalin-evoked nociceptive behaviour in SD and WKY rats over the full 60-minute testing period. * $p < 0.05$, ** $p < 0.01$ vs. SD counterpart, # $p < 0.01$ vs SD VEH (Tukey's). All data are expressed as mean \pm SEM ($n = 8-21$ per group). VEH: Vehicle Sprague-Dawley; WKY: Wistar-Kyoto.

Temporal analysis of the data in 5-minute time bins revealed that a biphasic formalin response was expressed by both SD and WKY VEH treated rats (Figure 7.5).

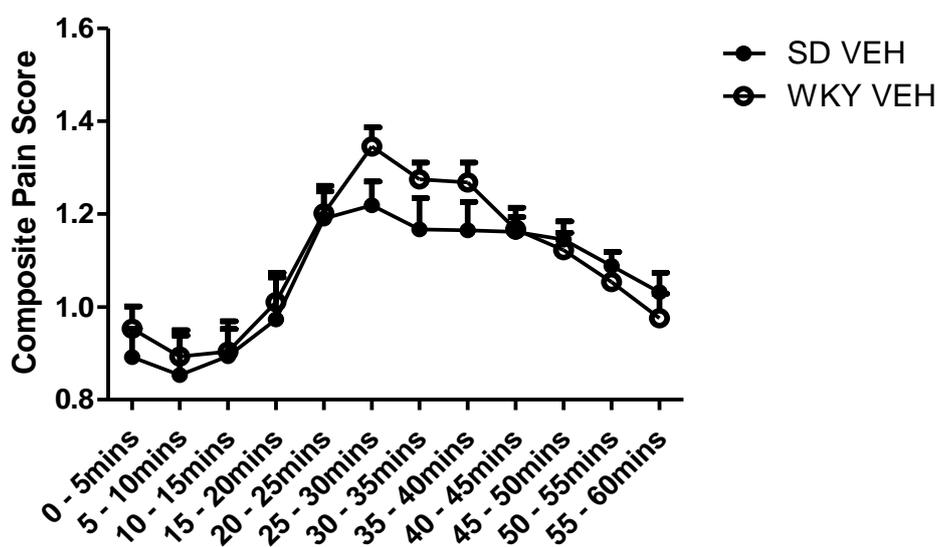


Figure 7.5: Formalin-evoked nociceptive behaviour in VEH-treated SD and WKY rats. Data are presented as mean \pm SEM, ($n = 17-21$ per group). SD: Sprague-Dawley; WKY: Wistar-Kyoto; VEH: Vehicle.

Temporal analysis of the data subdivided into 5-minute time bins and using 2-way repeated measures ANOVA revealed a significant main effect of strain ($F(1,104)=22.948$, $p<0.001$), treatment ($F(4,104)=8.467$, $p<0.001$) and strain*treatment ($F(4,104)=4.182$, $p<0.01$). Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated ($\chi^2(65)=377.107$, $p<0.001$) and therefore a Greenhouse-Geisser correction was used. There was a significant main effect of time ($F(5.83,600.70)=49.996$, $p<0.001$) and time*strain ($F(5.83,600.70)=3.437$, $p<0.01$) and time*treatment ($F(23.33,600.70)=1.667$, $p<0.05$) but not time*strain*treatment ($F(23.33,600.70)=1.104$, $p=0.334$). Post-hoc analysis revealed a significant reduction in formalin-evoked nociceptive behaviour in mAEA- and URB597-treated SD rats, an effect not seen in WKY rats (SD VEH vs. SD mAEA, 5-10mins, 10-15mins, $p<0.05$; 20-25mins, 25-30mins, $p<0.001$; SD VEH vs. SD URB597, 5-10mins, 10-15mins, 20-25mins $p<0.05$; 25-30mins, $p<0.01$; 30-35mins, $p<0.05$) (Figure 7.6).

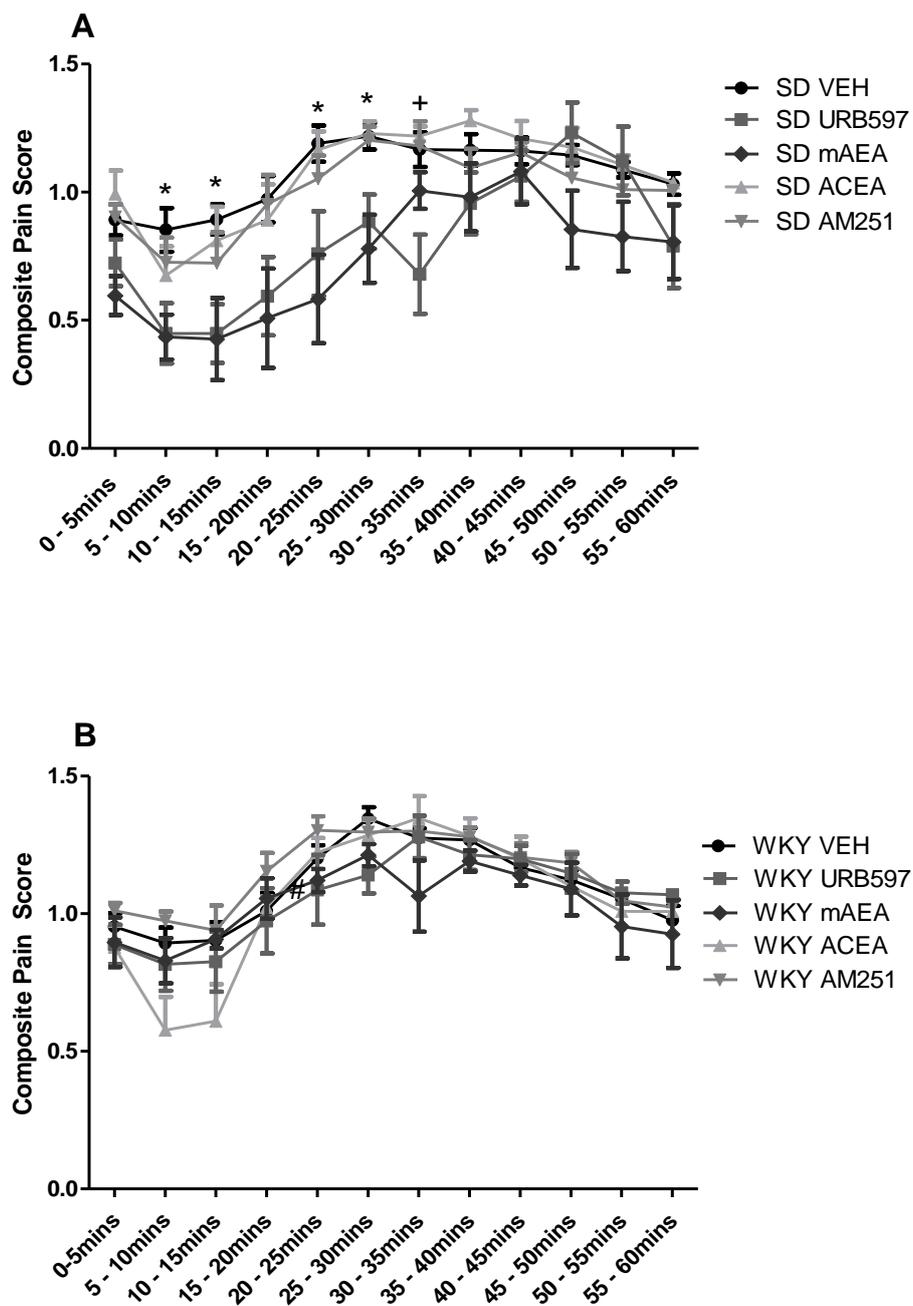


Figure 7.6: The effect of bilateral intra-IL microinjection of a CB₁ receptor agonist, antagonist, FAAH inhibitor or AEA analog on formalin-evoked nociceptive behaviour in (a) SD rats and (b) WKY rats. * $p < 0.05$ SD VEH vs. SD mAEA and SD URB597, + $p < 0.05$ SD VEH vs. SD URB597. Data are expressed as mean \pm SEM. (n=8-21 per group). SD: Sprague-Dawley; WKY: Wistar-Kyoto; VEH: Vehicle.

7.3.4 Effects of intra-IL administration of a CB₁ receptor agonist, antagonist, FAAH inhibitor or AEA analog, on general locomotor and exploratory behaviour during the formalin trial

Two-way ANOVA revealed a significant main effect of strain ($F(1,104)=127.142$, $p<0.001$), treatment ($F(4,104)=8.037$, $p<0.001$) and strain*treatment ($F(4,104)=5.281$, $p<0.001$) on the total distance moved in the arena. Post-hoc analysis revealed a significant increase in distance moved in SD URB597 and SD mAEA-treated rats compared to SD VEH-treated rats, effects not seen in WKY rats (SD VEH vs. SD URB597, $p=0.018$). WKY rats exhibited decreased distance moved compared to SD rats (SD VEH vs. WKY VEH, $p<0.001$), an effect maintained in all WKY treatment groups (Table 7.3).

Two-way ANOVA revealed a significant main effect of strain ($F(1,104)=31.731$, $p<0.001$), treatment ($F(4,104)=1.461$, $p=0.219$) but not strain*treatment ($F(4,104)=1.916$, $p=0.113$) on the duration of walking in the arena. Further post-hoc analysis revealed no significant effects between-groups (Table 7.3).

Two-way ANOVA revealed a significant main effect of strain ($F(1,104)=5.384$, $p<0.05$) but not treatment ($F(4,104)=0.897$, $p=0.468$) or strain*treatment ($F(4,104)=1.455$, $p=0.221$) on the duration of time spent rearing in the arena. Further post-hoc analysis revealed no significant effects between-groups (Table 7.3).

Two-way ANOVA revealed a significant main effect of strain ($F(1,104)=9.316$, $p<0.01$) but not treatment ($F(4,104)=0.290$, $p=0.884$) or strain*treatment ($F(4,104)=1.626$, $p=0.173$) on the amount of time spent grooming in the arena. Further post-hoc analysis revealed no significant effects between-groups (Table 7.3).

Kruskal Wallis comparisons revealed no significant effect ($\chi^2(9)=8.746$, $p<0.461$) for the amount of defecation in the arena (no. of pellets) over the course of the 60-minute testing period (Table 7.3).

Treatment Group	Distance Moved (cm)	Walking (s)	Rearing (s)	Grooming (s)	Defecation (number of pellets)
SD-VEH	3204±246	193±28	11±6	30±6	0(1)
SD-URB597	4628±458#	225±46	9±5	14±4	1(2)
SD-mAEA	4633±453#	183±19	13±7	24±9	1(2)
SD-ACEA	2947±360	179±23	1±1	31±17	1(1)
SD-AM251	2497±299	105±17	1±0	16±5	1(1)
WKY-VEH	1811±100*	112±21	1±0	34±5	1(1)
WKY-URB597	1757±131*	60±14	1±1	51±16	1(1)
WKY-mAEA	1853±115*	78±19	1±0	27±4	1(2)
WKY-ACEA	1508±84*	68±11	4±3	37±8	1(2)
WKY-AM251	1545±102*	90±19	1±1	41±8	1(2)

Table 7.3: Effects of intra-IL administration of a CB1 receptor agonist, antagonist, FAAH inhibitor or AEA analog on general locomotor activity and exploratory behaviours during the 60 minute formalin trial. * $p < 0.05$ vs. SD counterpart; # $p < 0.05$ vs. SD VEH. Data are presented as mean \pm SEM with the exception of defecation which is presented as median (interquartile range) (n=8-20, per group). (SD: Sprague-Dawley, WKY: Wistar-Kyoto).

7.3.5 Effect of Intra-IL administration of URB597 on AEA, OEA, PEA or 2-AG levels in the IL, PrL and ACC of formalin-treated SD and WKY rats

Infralimbic cortex

Two-way ANOVA revealed no significant effect of treatment ($F_{1,24}=2.397$, $p=0.137$), strain ($F_{1,24}=0.310$, $p=0.584$) or strain*treatment ($F_{1,24}=0.114$, $p=0.739$) on the levels of AEA in the IL (Figure 7.7).

Two-way ANOVA revealed no significant effect of strain ($F_{1,25}=0.112$, $p=0.741$), treatment ($F_{1,25}=1.788$, $p=0.196$) or strain*treatment ($F_{1,25}=0.231$, $p=0.636$) on the levels of 2-AG in the IL (Figure 7.7).

Two-way ANOVA revealed no significant effect of strain ($F_{1,22}=0.771$, $p=0.392$), treatment ($F_{1,22}=0.123$, $p=0.730$) or strain*treatment ($F_{1,22}=0.178$, $p=0.678$) on the levels of PEA in the IL (Figure 7.7).

Two-way ANOVA revealed no significant effect of strain ($F(1,24)=3.126$, $p=0.092$), treatment ($F(1,24)=0.645$, $p=0.431$) or strain*treatment ($F(1,24)=3.438$, $p=0.077$) on the levels of OEA in the IL (Figure 7.7).

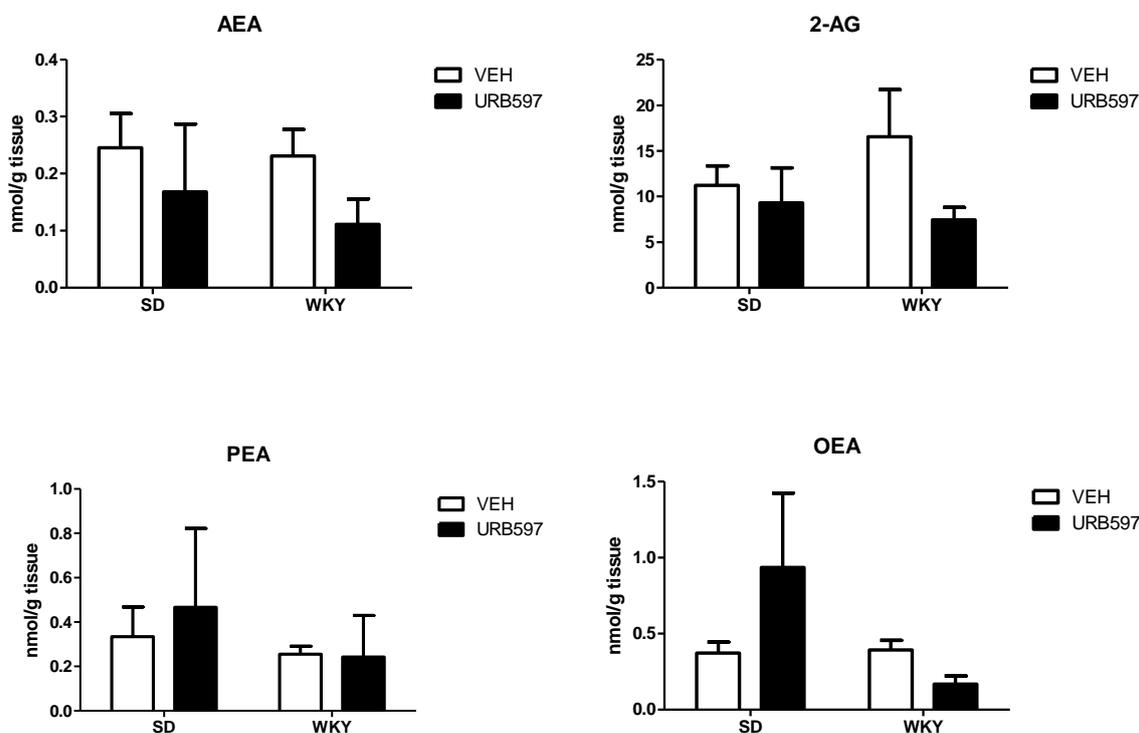


Figure 7.7: Effect of bilateral microinjection of the FAAH inhibitor URB597 on AEA, OEA, PEA or 2-AG levels in the IL of formalin-treated SD and WKY rats. All data are expressed as mean \pm SEM ($n=4-8$ per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; VEH, Vehicle.

Prelimbic cortex

Two-way ANOVA revealed a significant main effect of treatment ($F(1,26)=9.317$, $p<0.01$) but not strain ($F(1,26)=0.485$, $p=0.494$) or strain*treatment ($F(1,26)=0.107$, $p=0.746$) on the levels of AEA in the PrL. Post-hoc analysis revealed no significant group changes (Figure 7.8).

Two-way ANOVA revealed a significant main effect of strain ($F(1,24)=9.505$, $p<0.01$) but not treatment ($F(1,24)=6.637$, $p=0.055$) or strain*treatment ($F(1,24)=0.000$, $p=0.999$) on the levels of 2-AG in the PrL. Post-hoc analysis revealed no significant group changes (Figure 7.8).

Two-way ANOVA revealed no significant effect of strain ($F(1,25)=1.498$, $p=0.235$), treatment ($F(1,25)=2.345$, $p=0.141$) or strain*treatment ($F(1,25)=0.002$, $p=0.963$) on the levels of PEA in the PrL (Figure 7.8).

Two-way ANOVA revealed no significant effect of strain ($F(1,26)=1.134$, $p=0.298$), treatment ($F(1,26)=0.713$, $p=0.408$) or strain*treatment ($F(1,26)=0.773$, $p=0.389$) on the levels of OEA in the PrL (Figure 7.8).

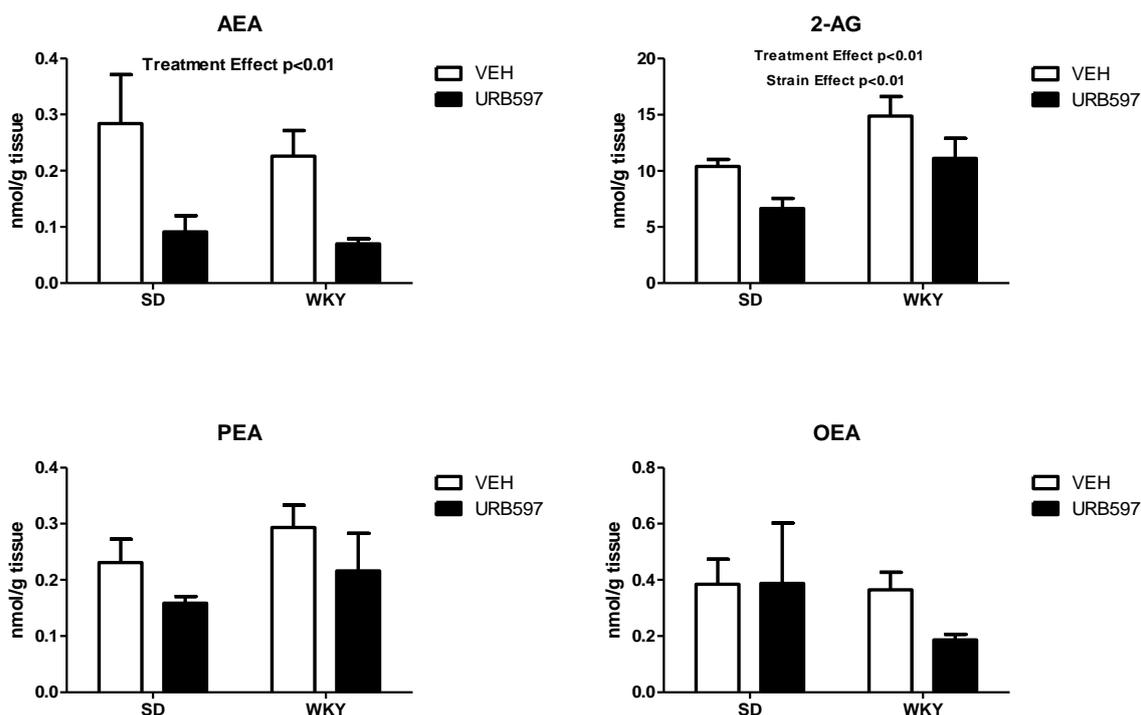


Figure 7.8: Effect of bilateral microinjection of the FAAH inhibitor URB597 on AEA, OEA, PEA or 2-AG levels in the PrL of formalin-treated SD and WKY rats. All data are expressed as mean \pm SEM ($n=4-8$ per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; VEH, Vehicle.

Anterior cingulate cortex

Two-way ANOVA revealed a significant main effect of treatment ($F(1,26)=17.461$, $p<0.001$) but not strain ($F(1,26)=0.571$, $p=0.458$) or strain*treatment ($F(1,26)=0.719$, $p=0.406$) on the levels of AEA in the ACC. Further post-hoc analysis revealed a significant decrease in the levels of AEA upon URB597 treatment in WKY rats, an effect not reaching significance in SD rats (Figure 7.9).

Two-way ANOVA revealed a significant main effect of treatment ($F(1,26)=7.356$, $p<0.05$) and strain*treatment ($F(1,26)=5.270$, $p<0.05$) but strain alone failed to reach significance ($F(1,26)=4.182$, $p=0.053$) for the levels of 2-AG in the ACC. Further post-hoc analysis revealed significantly higher levels of 2-AG in WKY rats compared to SD rats (SD VEH vs. WKY VEH, $p<0.05$). Treatment with URB597 lowered the levels of 2-AG in WKY rats compared to VEH-treated counterparts (WKY VEH vs. WKY URB597, $p<0.01$) (Figure 7.9).

Two-way ANOVA revealed no significant main effect of strain ($F(1,25)=0.382$, $p=0.543$), treatment ($F(1,25)=3.404$, $p=0.079$) or strain*treatment ($F(1,25)=0.016$, $p=0.900$) on the levels of PEA in the ACC (Figure 7.9).

Two-way ANOVA revealed a significant main effect of treatment ($F(1,25)=13.979$, $p<0.001$) but not strain ($F(1,25)=0.348$, $p=0.561$) or strain*treatment ($F(1,25)=0.655$, $p=0.427$) on the levels of OEA in the ACC. Further post-hoc analysis revealed a significant decrease in OEA levels in WKY rats upon URB597 treatment, an effect not seen in SD rats (WKY VEH vs WKY URB597, $p<0.05$) (Figure 7.9).

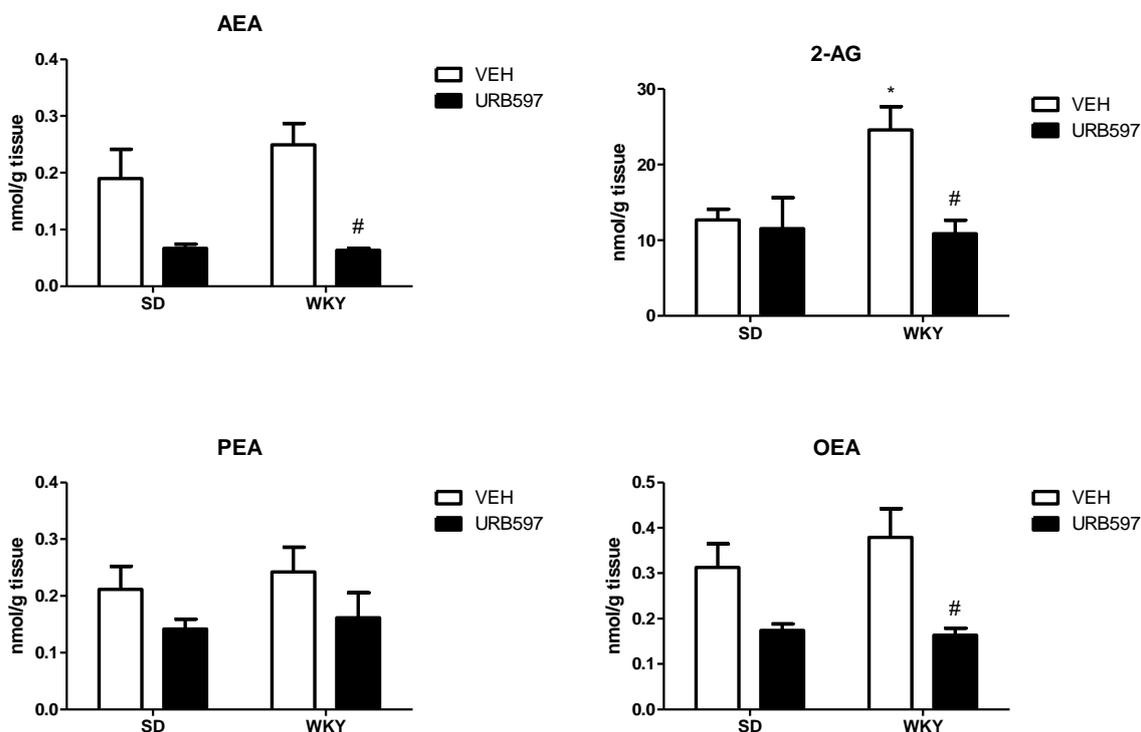


Figure 7.9: Effect of bilateral microinjection of the FAAH inhibitor URB597 on AEA, OEA, PEA or 2-AG levels in the ACC of formalin-treated SD and WKY rats. # $p < 0.05$ vs VEH; * $p < 0.05$ vs SD. All data are expressed as mean \pm SEM (n=4-8 per group). SD, Sprague Dawley; WKY, Wistar-Kyoto; VEH, Vehicle.

7.3.6 CB₁ receptor protein expression in the IL, PrL and ACC of SD and WKY rats

The results from the t-test revealed that there was no significant difference in the expression levels of CB₁ in the IL ($t(13)=0.304$, $p=0.766$), PrL ($t(13)=0.285$, $p=0.780$) or ACC ($t(13)=0.735$, $p=0.476$) of SD and WKY rats (Figure 7.10).

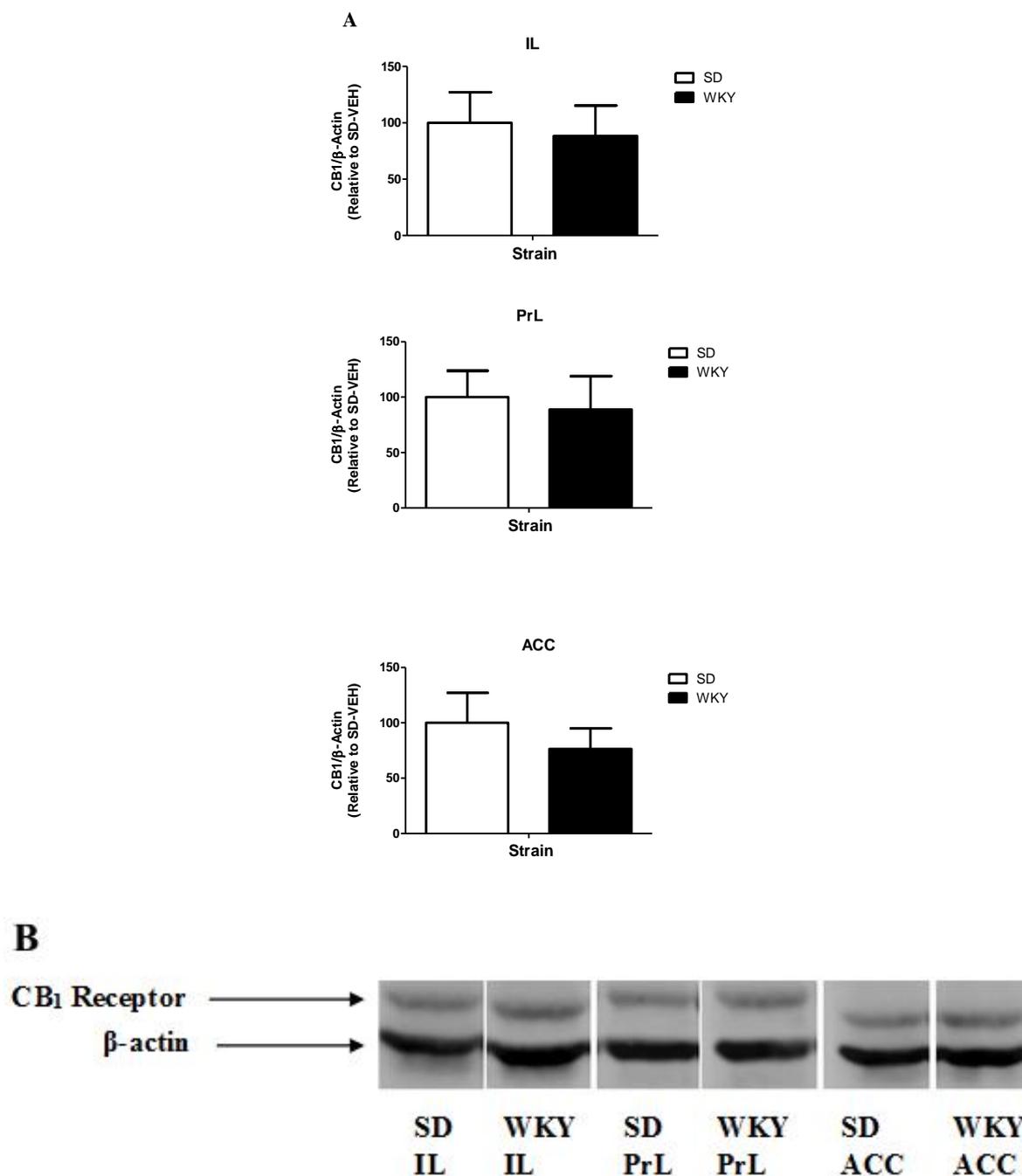


Figure 7.10: CB₁ receptor protein expression levels in the IL, PrL and ACC in vehicle-treated SD and WKY rats (A). Data are expressed as mean \pm SEM (n=8-9 per group). Representative western immunoblot image (B), showing the bands for CB₁ receptor (63kDa) and β -actin (42kDa) for each of the six groups. (SD: Sprague-Dawley, WKY: Wistar-Kyoto, IL: Infralimbic cortex, PrL: Prelimbic cortex, ACC: anterior cingulate cortex, PFC: Prefrontal cortex).

7.4 Discussion

In the present study, WKY rats receiving intra-IL injection of vehicle did not exhibit greater formalin-evoked nociceptive behaviour than SD counterparts. In SD rats, intra-IL administration of mAEA and URB597 reduced formalin-evoked nociceptive behaviour, effects not observed in WKY rats. Intra-IL administration of AM251 and ACEA had no effect on formalin-evoked nociceptive behaviour in either SD or WKY rats. Intra-IL administration of URB597 did not alter the levels of AEA, 2-AG, PEA and OEA in the IL and PrL of SD rats. WKY rats exhibited higher levels of 2-AG in the ACC compared to SD rats. Intra-IL administration of URB597 significantly reduced the levels of AEA, 2-AG and OEA in the ACC of WKY rats. There were no significant strain related differences in the expression levels of CB₁ in the IL, PrL and ACC of SD and WKY rats. The results from the present study contrast with previous studies from our own and other groups demonstrating a robust increase in formalin-evoked nociceptive behaviour in WKY rats versus SD counterparts. Moreover, the results indicate strain differences in the endocannabinoid modulation of nociceptive responding in the IL as mAEA and URB597 in the IL decreased formalin-evoked nociceptive behaviour in SD but not WKY rats.

The present study found no significant difference in formalin-evoked nociceptive behaviour between SD and WKY VEH treated rats. This contrasts previous work in the laboratory where naïve WKY rats displayed increased formalin-evoked nociceptive behaviour compared to naïve SD rats (Burke et al., 2010, Rea et al., 2014b, Madasu et al., 2016, Okine et al., 2017, Jennings, 2015b, Jennings et al., 2015). This also contrasts the work presented in chapter 6 where both FC and NFC WKY rats displayed increased formalin-evoked nociceptive behaviour compared to SD rats. This implies a between-study variability in WKY rats that may account for the lack of hyperalgesia seen in the present chapter. It is important to note that the formalin-induced nociceptive behaviour exhibited by SD rats in the present study is higher than in others previously obtained during a 60-minute testing period and this is likely masking any strain-related hyperalgesic effects between SD and WKY rats (Madasu et al., 2016, Jennings et al., 2015, Burke et al., 2010, Rea et al., 2014b). The SD rats used in the present study were sourced from Harlan (now Envigo), similar to the studies mentioned above (Madasu et al., 2016, Jennings et al., 2015, Rea et al., 2014b). At the time of the study presented in this chapter, Harlan had moved their SD breeding colony from the UK to Italy and so although the rats are from the same supplier, the change in breeding colony location (UK vs. Italy) from that used in previous studies may have influenced the formalin-evoked nociceptive behaviour exhibited

by SD rats in this study. The SD rats used in this chapter are sourced from the same breeding colony as chapter 6. In chapter 6 we see a clear hyperalgesic phenotype in WKY rats compared to SD rats and so it is possible that the change in breeding colony did not contribute to the hyperalgesia seen in SD rats in this chapter. It is possible that cannulation of the IL could have caused a higher CPS in SD rats without effecting WKY responding. Despite the lack of hyperalgesia seen in the WKY rat strain compared to SD rats, strain-related differences upon treatment with endocannabinoid related drugs were still observed indicating altered EC-modulation of nociceptive tone between SD and WKY rats.

Intra-IL administration of mAEA or URB597 significantly reduced formalin-evoked nociceptive behaviour in SD but not WKY rats. URB597 is a FAAH inhibitor, blocking the degradation of AEA, PEA and OEA and thus increasing the endogenous tone of these ligands. These results suggest that increasing FAAH substrate/AEA tone in the IL of the mPFC is antinociceptive in SD but not WKY rats. However, this does not correlate with our mass spectrometry data (discussed later in the discussion) that indicate that intra-IL URB597 does not increase the levels of AEA, PEA or OEA in either SD or WKY rats, although this may be due to some methodological issues in the mass spectrometry data. Systemic administration of mAEA is antinociceptive in the tail-immersion test, hot-plate test and writhing test in Wistar rats (Korossy-Mruk et al., 2013). Similarly, systemic URB597 is antinociceptive in the acid-stimulated stretching test in SD rats (Kwilasz et al., 2014a) and attenuates mechanical allodynia, and acetone-induced cold allodynia in mice subjected to CCI (Kinsey et al., 2009). URB597 has also been shown to decrease thermal hyperalgesia in mice suffering from collagen-induced arthritis (Kinsey et al., 2011a). Furthermore, depending on the dose, intra-vIPAG URB597 enhanced or attenuated nociception in the plantar test of rats via TRPV1 and CB₁ receptors respectively (Maione et al., 2006). The results from the present study are comparable to those of de Novellis et al., who show that URB597 decreases SNI-induced allodynia when injected into the PrL/IL cortex (de Novellis et al., 2011a). In contrast to the study presented here, Rea et al., found that intra-IL URB597 had no effect on formalin-evoked nociceptive behaviour in NFC Lister-Hooded rats (Rea et al., 2018), however Rea et al., only looked at formalin-evoked nociceptive behaviour in the second phase (30-60 minutes post administration) of the formalin test. The results presented in this chapter indicate antinociceptive effects of URB597 in the first half (5-35 minutes) of the formalin test. Taken together, these studies imply possible region-specific effects of FAAH substrates on nociceptive responding in rodents. Intra-ACC PEA (FAAH substrate) significantly attenuated

the first and early second phases of formalin-evoked nociceptive behaviour in SD rats, an effect mediated by a possible AEA-induced activation of CB₁ receptors (Okine et al., 2016). It is possible that the URB597 effect we see in SD rats is mediated by increased endogenous tone of both PEA and AEA. Further studies testing intra-IL administration of PEA would be required to determine this. Unlike SD rats, WKY rats do not display reduced formalin-evoked nociceptive behaviour upon treatment with either mAEA or URB597. In comparison, systemic administration of URB597 has been shown to reduce the increase in formalin-evoked nociceptive behaviour exhibited by WKY rats, an effect attenuated by CB₁ receptor blockade in the RVM (Rea et al., 2014b). Previous studies have shown that WKY rats express higher levels of FAAH and lower levels of AEA in the frontal cortex (Vinod et al., 2012). It is possible that treatment with URB597 in this rat strain at the dose used in this study is not enough to fully block FAAH enzyme activity due to the higher levels of FAAH exhibited in WKY rats. Further studies co-administering mAEA and URB597 would be useful to determine if a dual approach can reduce formalin-evoked nociceptive behaviour in WKY rats.

Intra-IL administration of the CB₁ receptor agonist ACEA had no effect on formalin-evoked nociceptive behaviour in either SD or WKY rats. This result contrasts with previous studies showing antinociceptive effects of ACEA at the same dose in the IPAG and dIPAG in SD rats following intra-plantar injection of formalin but is comparable to another where intra-vIPAG ACEA at the same dose had no effect on formalin-evoked nociceptive behaviour (Jennings, 2015b). Together these studies imply a possible region-specific effect of ACEA supraspinally. ACEA is a potent CB₁ receptor agonist but can potentially activate other receptors such as TRPV1, with a lower efficacy and potency (Smart et al., 2000). In both pain and anxiety paradigms, CB₁ and TRPV1 can have opposing effects; at low activating doses CB₁ tends to be activated producing antinociceptive and anxiolytic effects while at high activating doses TRPV1 tends to produce pro-nociceptive and anxiogenic effects (Rubino et al., 2008b, Casarotto et al., 2012, Palazzo et al., 2008). Upon administration into the IL it is possible that ACEA dually activates both CB₁ and TRPV1 and thus due to their opposing effects ACEA produces no effect on formalin-evoked nociceptive behaviour. In order to test this hypothesis, co-administering ACEA with a TRPV1 and CB₁ antagonist would be necessary. As with ACEA, AM251 also had no effect on formalin-evoked nociceptive behaviour. This is comparable to the intra-vIPAG AM251 which did not affect formalin-evoked nociceptive behaviour in either strain, although intra-IPAG AM251 increases formalin-evoked nociceptive behaviour in both SD and WKY rats and intra-dIPAG AM251 increases formalin-evoked

nociceptive behaviour in SD but not WKY rats (Jennings, 2015b). In comparison to the lack of effect of intra-IL AM251 in the current study, intra-ACC administration of AM251 has been shown to reduce formalin-evoked nociceptive behaviour in SD rats (Okine et al., 2016). Furthermore, intra-IL/PrL administration of AM251 had no effect on SNI-induced allodynia in rats (de Novellis et al., 2011a). Likewise, Rea et al., found that intra-IL AM251 had no effect on formalin-evoked nociceptive behaviour in NFC Lister-Hooded rats, however in this study formalin-evoked pain behaviour was only scored during the second phase of formalin (Rea et al., 2018). In comparison, systemic administration of AM251 has been shown to potentiate nociceptive responding in WKY rats following formalin administration, while having no effect in SD rats. Similarly, intra-RVM AM251 attenuated an antinociceptive effect induced via FAAH blockade with URB597 (Rea et al., 2014b). It is possible that CB₁ receptors in the IL do not have any effect on formalin-evoked nociceptive behaviour in rats and mAEA and URB597 may be exerting their effects via a non-CB₁ receptor. Other AEA targets could include CB₂ (Griffin et al., 2000b, Felder et al., 1996, Petrosino and Di Marzo, 2017), TRPV1 (De Petrocellis et al., 2001a, Di Marzo et al., 2001, Ross et al., 2001, Smart et al., 2000), PPARs (LoVerme et al., 2005, O'Sullivan and Kendall, 2010, Pistis and O'Sullivan, 2017, Okine et al., 2014) and GPR55 (Pertwee, 2007, Sharir and Abood, 2010, Ryberg et al., 2007, Kramar et al., 2017). As mAEA and URB597 were not co-administered with a CB₁ receptor antagonist, antinociceptive effects via this receptor cannot be ruled out. Co-administering mAEA and URB597 with an antagonist for each of the receptors above along with a CB₁ antagonist could address this question.

WKY rats displayed lower distance moved than SD rats in both the pre-formalin and formalin testing periods. This is comparable to previous studies showing hypo-locomotion in WKY rats in the formalin test and open field test (Drolet et al., 2002, Ferguson and Cada, 2003, Nosek et al., 2008, Burke et al., 2010, Jennings, 2015b). Moreover, mAEA, URB597, ACEA and AM251 had no effect on locomotor activity during the pre-formalin testing period implying that the decrease in formalin-evoked nociceptive behaviour was not due to locomotor impairment induced by the drugs. Furthermore, during the 60-minute testing period mAEA- and URB597-treated SD rats exhibited increased distance moved compared to VEH-treated SD rats, providing more evidence that the decreased pain behaviour in this strain is not due to locomotor deficits. The increase in locomotor activity in this strain may be due to the decreased pain behaviour upon treatment with mAEA and URB597. Comparable to the pain behaviour seen, mAEA and URB597 treated WKY rats did not exhibit increased distance moved during

the 60-minute formalin trial. There was no effect of drug or strain on walking, rearing, grooming or defecation. Paw oedema developed similarly in both strains and all treatment groups.

Treatment with URB597 did not increase the levels of AEA, PEA, OEA and 2-AG in the IL of SD or WKY rats. This is comparable to previous studies demonstrating that URB597 injected directly in the subregions of the mPFC does not increase the levels of these ligands (see chapter 2 and Rea et al., 2018). In comparison, URB597 has been shown to increase the levels of AEA in the brain when administered systemically or directly into the vIPAG (Maione et al., 2006, Flannery et al., 2017, Kerr et al., 2012). The levels of AEA, PEA, OEA and 2-AG are measured 70-minutes after URB597 microinjection. It is possible that URB597 increased the levels of AEA and related n-acylethanolamines during the first phase of the formalin trial where we see the decrease in formalin-evoked nociceptive behaviour, but these levels return to baseline levels by the time we measure the levels 70-minutes post microinjection. This is likely as we are not seeing any behavioural effects by the end of the testing period. In order to test this, the levels of these cannabinoids should be measured 30-minutes post formalin when there are still robust behavioural effects. Moreover, the n number for URB597 treated rats is very low for the mass spectrometry data (n=4). This may have led to large error and decreased power in the data. The low n numbers were due to a methodological issue and human error during processing of the samples.

In comparison to the IL, there were trends for decreased levels of AEA and 2-AG in the PrL of SD and WKY rats following intra-IL administration of URB597. Similarly, there was a significant increase in the levels of 2-AG in the ACC of WKY rats and significant or trends for a decrease in the levels of AEA and PEA in the ACC of SD and WKY rats following intra-IL URB597. It is possible that by inhibiting FAAH in the IL, FAAH activity is increased in the PrL and ACC of SD and WKY rats to compensate for the decreased activity in the IL. Moreover, the levels of 2-AG are increased in the ACC of VEH treated WKY rats compared to SD rats. WKY rats exhibit a stress hyper-responsive phenotype and 2-AG has previously been shown to be upregulated upon exposure to stress (Lutz et al., 2015, Morena et al., 2015, Patel et al., 2009). Indeed, in chapters 3-5 increasing the endogenous tone of 2-AG via MAGL inhibition increased formalin-evoked nociceptive behaviour in FC rats, i.e. increased pain related behaviour in stressed rats. It is possible that the increased 2-AG tone in the PrL and ACC in WKY rats may be acting as both a coping mechanism to deal with their anxiogenic phenotype while also contributing to their co-morbid hyperalgesic phenotype. The increased

levels of 2-AG are decreased in WKY rats upon URB597 treatment. This may be due to a URB597-mediated increase in AEA which then down-regulates 2-AG tone.

In order to determine if the strain related differences seen with SD and WKY rats treated with mAEA and URB597 were due to CB₁ receptor related differences, western blotting analysis was carried out to determine the levels of CB₁ in the IL, PrL and ACC of SD and WKY rats. The results show that regardless of the region there is no difference in the levels of CB₁ expression in the mPFC of SD compared to WKY rats. This result implies that the effects of mAEA and URB597 were not due to differences in receptor expression levels in the IL of SD and WKY rats but this does not rule out a receptor activity difference between rat strains. It is possible that WKY rats have dysfunctional CB₁ receptors and this the reason we see a decrease in pain related behaviour in SD but not WKY rats. Alternatively, it could be that mAEA and URB597 are acting at a non-CB₁ related receptor, as discussed above. An antagonist-based study, as described above, paired with western blotting to determine receptor expression differences between SD and WKY would likely shed some light on these strain related differences in pain processing.

In conclusion, these findings provide evidence for antinociceptive activity of AEA and FAAH substrates in the IL of SD, but not WKY, rats following intra-plantar injection of formalin. The results suggest a strain difference in the endocannabinoid system function in the IL between SD and WKY rats, a key neuroanatomical brain region involved in descending pain modulation. The mechanism by which mAEA and URB597 exert their effects remain to be determined and further studies are required to uncover the receptor mechanism by which these drugs act.

Chapter 8 : General Discussion

Chronic pain remains a major unmet clinical problem affecting ~25% of the population and costing the economy immensely (McBeth and Jones, 2007). Treatment of chronic pain is difficult, with physicians tending to focus on management rather than elimination of pain. The PFC is believed to play a key role in the integration and regulation of information necessary to allow for the selection of an appropriate behavioural response (i.e. executive control) (Miller and Cohen, 2001). Patients with chronic pain or fear-related disorders exhibit functional and structural abnormalities within their mPFC (Baliki et al., 2006b, Baliki et al., 2014, Mansour et al., 2013, Yoon et al., 2013, Kucyi et al., 2014). Different forms of stress or aversion affect pain processing in a differential manner, either enhancing or inhibiting pain. Moreover, the relationship between pain and fear, and the neurobiological mechanisms underlying their interaction, are incompletely understood. FCA relies on the robust activation of the body's endogenous analgesic system. Understanding the neurochemical and molecular mechanisms mediating such potent endogenous analgesia and the role that the mPFC plays may facilitate the development of novel therapeutic approaches and improve the quality of life of patients suffering from pain disorders.

The work described in this thesis examined the role of the endocannabinoid system within the mPFC in FCA, contextual fear in the presence of nociceptive tone and formalin-evoked nociceptive behaviour. I also explored the influence of genetic background on FCA using the stress- and pain-hyperresponsive WKY rat. This discussion will focus on appraising the most significant behavioural, pharmacological and neurobiological findings and discuss how these contribute to our understanding of the neurobiology underpinning pain, fear and FCA. It will also consider some limitations to the work described and highlight some areas worthy of future investigation.

The major findings of the work described in this thesis are:

- (1) FAAH inhibition and CB₁ receptor antagonism in the ACC have no effect on the expression of formalin-evoked nociceptive behaviour, contextual fear and FCA in rats.
- (2) MAGL inhibition in the IL, PrL or ACC attenuates the expression of FCA without affecting the expression of formalin-evoked nociceptive behaviour or conditioned fear in the presence of nociceptive tone. These effects were not mediated by CB₁ receptors in these subregions of the mPFC.

- (3) FCA is attenuated by a 2-AG-CB₂ receptor signalling mechanism in the ACC. Pharmacological blockade of CB₂ receptors in the ACC also reduced formalin-evoked nociceptive behaviour in NFC rats and prolonged the duration of freezing in FC rats, suggesting that CB₂ receptors in the ACC may facilitate formalin-evoked nociceptive behaviour and terminate fear-related behaviour.
- (4) Compared to SD rats, WKY rats exhibit reduced expression of FCA and contextual fear, and greater formalin-evoked nociceptive behaviour, effects associated with reduced levels of AEA in the IL.
- (5) In SD rats, intra-IL administration of mAEA or the FAAH inhibitor URB597 reduced formalin-evoked nociceptive behaviour, an effect not seen in WKY rats. Moreover, intra-IL administration of the CB₁ receptor agonist ACEA or the CB₁ antagonist AM251 had no effect on formalin-evoked nociceptive behaviour in either SD or WKY rats.

In chapter 2, I studied the role of FAAH substrates in the ACC on formalin-evoked nociceptive behaviour, contextual fear and FCA in rats. The findings from this study demonstrate that neither FAAH inhibition nor pharmacological blockage of CB₁ receptors, alone or in combination, had any effect on formalin-evoked nociceptive behaviour, contextual fear or FCA in rats. In contrast, previous studies have demonstrated that systemic (Finn et al., 2004, Olango et al., 2014, Guhring et al., 2002), intra-BLA (Roche et al., 2007), intra-dlPAG (Olango et al., 2012) or intra-IL/PrL (Rea et al., 2018) administration of a CB₁ receptor antagonist attenuates expression of FCA in rats. Moreover, systemic, intra-PrL or intra-ventral hippocampus administration of URB597 has been shown previously to enhance/prolong the duration of FCA (Butler et al., 2012, Butler et al., 2008, Ford et al., 2011, Rea et al., 2018) while intra-IL URB597 attenuates the expression of FCA (Rea et al., 2018).

FAAH substrates within the mPFC subregions may be an important factor contributing to the differential regulation of fear- and pain-related behaviour by these subregions. Alterations in FAAH substrate levels, coupled with differences in circuitry within, and projections to and from, each of these three mPFC subregions (Vertes, 2004, Hoover and Vertes, 2007, Vertes, 2002), likely underlies the different roles these subregions play in fear, pain and FCA. One of the first reports of specific and differential functions of subregions in the mPFC was by Vidal-Gonzalez and colleagues (2006). They found that microstimulation of the PrL increased the expression of conditioned fear to a tone and prevented extinction, while microstimulation of the IL reduced the expression of conditioned fear and microstimulation of the ACC had no

effect on either expression or extinction of conditioned fear (Vidal-Gonzalez et al., 2006). Inactivation of the PrL but not IL depressed fear responses while inactivation of the IL but not PrL impaired the consolidation and retrieval of fear extinction in rats (Laurent and Westbrook, 2009). Lesioning (Kim et al., 2013) or pharmacological inactivation (Sierra-Mercado et al., 2011, Corcoran and Quirk, 2007) of the PrL impairs the expression of conditioned fear without affecting extinction, while inactivation of the IL has no effect on fear expression but impairs the acquisition of extinction as well the extinction memory (Sierra-Mercado et al., 2011). Fewer studies have compared the respective roles of these mPFC regions in modulating pain. Pre- and post-conditioning muscimol-mediated inactivation of IL and PrL had no effect on expression of formalin-evoked pain *per se*, but differentially affected formalin-evoked condition place aversion which was impaired by PrL, but not IL, inactivation (Jiang et al., 2014). A noteworthy point is that these studies tended to only look at the differences between the IL and PrL, and did not focus on the ACC. The ACC is in a pivotal position to influence neuronal activity in the IL and PrL, and despite the negative findings in chapter 2 for FAAH inhibition in the ACC, a role for additional components of the endocannabinoid system (e.g. MAGL/2-AG/CB₂) within the ACC, IL and PrL in the regulation of conditioned fear, pain and FCA cannot be ruled out and was the focus of subsequent chapters.

In chapters 3-5, I studied the effects of MAGL inhibition in the IL, PrL and ACC on formalin-evoked nociceptive behaviour, contextual fear and FCA in rats. MAGL is the primary enzyme catalysing the catabolism of 2-AG (Baggelaar et al., 2018). 2-AG regulates a wide array of physiological processes, including those underpinning, cognition, energy balance, pain sensation, fear and neuroinflammation. Moreover, brain 2-AG levels are ~170 times higher than those of AEA (Stella et al., 1997). In the IL, PrL and ACC, MAGL inhibition attenuated the expression of FCA in the experiments described herein. MAGL inhibition in these regions had no effect on the expression of contextually induced freezing in the presence of formalin-evoked nociceptive tone. These findings suggest that in the IL, PrL, and ACC, the expression of FCA is suppressed by MAGL substrates and that effects on FCA are not due to overt effects on either conditioned fear or formalin-evoked nociceptive behaviour. The results provide even more evidence (Rea et al., 2018) that these regions are important neural substrates regulating the expression of FCA and fear-pain interactions, alongside the ventral hippocampus (Ford et al., 2011), dIPAG (Olango et al., 2012) and the BLA (Rea et al., 2013, Roche et al., 2007). Moreover, these data are the first to demonstrate that the ACC is an important neural substrate regulating the expression of FCA and fear-pain interactions. The effect of the MAGL inhibitor

MJN110 on FCA in these regions was not blocked by co-administration with AM251. These results suggest that MAGL substrates modulate the expression of FCA via a non-CB₁ receptor-mediated mechanism. Putative targets could include CB₂ (Gonsiorek et al., 2000, Sugiura et al., 2000), GABA_A (Sigel et al., 2011), PPARs (Bouaboula et al., 2005), adenosine A₃ (Lane et al., 2010), TRPV1 (Yusaku et al., 2008) and GPR55 (Ryberg et al., 2007). In our follow-up study, in the ACC, MJN110-induced attenuation of the expression of FCA was blocked by co-administration with the CB₂ receptor antagonist AM630. This result suggests that 2-AG acts via CB₂ to attenuate FCA. This is the first study to provide evidence for a role of CB₂ in the ACC in FCA. Intra-ACC AM630 alone had no effect on the expression of FCA but did potentiate contextually induced freezing, an effect unopposed by co-administration with MJN110. These results suggest a role for CB₂ receptors independent of 2-AG levels in the ACC in the termination of fear-related behaviour. Intra-ACC administration of AM630 decreased formalin-evoked nociceptive behaviour in NFC rats, an effect blocked upon co-administration with MJN110. This finding provides evidence that CB₂ receptors in the ACC may facilitate the expression of formalin-evoked nociceptive behaviour in rats, an effect dependent on the levels of 2-AG in the ACC.

As previously mentioned, our group (Rea et al., 2018) and chapter 2 of this thesis have investigated the effects of FAAH inhibition and CB₁ receptor blockade in the subregions of the mPFC. In the IL, FAAH inhibition attenuated FCA, in the PrL FAAH inhibition prolonged the expression of FCA while FAAH inhibition had no effect in the ACC. CB₁ receptor blockade in the IL and PrL attenuated FCA, but had no effect in the ACC (Rea et al., 2018 and Chapter 2). While Rea et al., found that the FAAH inhibition via URB597 had differential effects in the subregions of the mPFC (IL, PrL and ACC), the results of chapter 3-5 show that regardless of the subregion, microinjection of the MAGL inhibitor MJN110 into the mPFC attenuates FCA. These results suggest that while FAAH inhibition may have differential, subregion-specific effects on FCA, the effect of MAGL inhibition remains consistent across the 3 subregions of the mPFC. Moreover, depending on the subregion, FAAH substrates and 2-AG may have the same or opposing roles (see Tables 8.1 and 8.2). Co-administration of a CB₁ receptor antagonist attenuated the effects of the FAAH inhibitor in the PrL, but not in the IL, suggesting that while FAAH substrates (possibly AEA) may act at CB₁ in the PrL to regulate FCA, their effects in the IL may be mediated by a non CB₁ receptor-mediated mechanism. This is comparable to the results described herein where 2-AG is acting via a non-CB₁ receptor target and highlight similarities and differences in the mechanisms behind the FAAH substrate and 2-AG

mechanisms of action in these regions. These results also indicate differential roles for FAAH substrates and 2-AG in the ACC in FCA. FAAH substrates in the ACC appear not to modulate the expression of FCA, while 2-AG is involved in the attenuation of FCA. As CB₂ antagonism attenuates the effect of MAGL inhibition in the ACC, it is possible that CB₂ is also mediating the effects of MAGL inhibition in the IL and PrL. Moreover, it is possible that the FAAH substrate-mediated effects in the IL are mediated by CB₂. Further studies are warranted to determine the precise receptor-related mechanisms by which these substrates exert their actions in these subregions of the mPFC.

Intra-IL, PrL or ACC administration of MJN110 alone or in combination with AM251 had no effect on the expression of contextually induced freezing behaviours in FC rats, despite attenuating FCA. These findings corroborate previous studies from our laboratory (Finn et al., 2004, Rea et al., 2013, Roche et al., 2010, Roche et al., 2007) and those of others (Helmstetter and Fanselow, 1987, Kinscheck et al., 1984), demonstrating that FCA can be altered independently of the expression of fear-related freezing in the presence of nociceptive tone. In contrast, Rea et al. (2018) and chapter 2 found that either URB597 or AM251 alone or in combination attenuated contextually induced freezing behaviour when injected into the IL indicating that the effects of endocannabinoid system modulation on FCA and contextually induced fear behaviour were not dissociable. Thus, while FAAH and MAGL inhibition in the IL both attenuate the expression of FCA, they seem to have differing effects on the expression of fear-related behaviours. Moreover, the effects of URB597 and AM251 in the PrL on FCA and contextually induced freezing were dissociable (Rea et al., 2018). This suggests that while having opposing roles in the expression of FCA (enhancing and attenuating respectively), FAAH and MAGL inhibition have similar effects in the PrL on the expression of contextually induced freezing in the presence of nociceptive tone.

The exact mechanism of action of MAGL substrates in the IL, PrL and ACC remains elusive. The data presented in Chapter 5 suggest that elevated levels of 2-AG arising from MAGL inhibition act via CB₂ in the ACC to attenuate FCA. It is possible that this mechanism also operates in the IL and PrL, but further studies are required to determine if this is the case. den Boon et al., have found that CB₂ receptors are expressed intracellularly and postsynaptically in the mPFC and decrease neuronal excitability (den Boon et al., 2012, den Boon et al., 2014). In the ACC, it is possible that 2-AG acts via postsynaptic CB₂ receptors to decrease neuronal excitability and attenuate FCA. This could also be the case in the IL and PrL. In particular, in the IL, both MAGL inhibition and CB₁ receptor antagonism attenuate FCA. In the mPFC, CB₁

receptors are primarily expressed on presynaptic GABAergic interneurons facing postsynaptic glutamatergic neurons. It is possible that CB₁ receptor antagonism alone causes an increased GABA influence in the mPFC which decreases excitability of glutamatergic neurons and attenuates the expression of FCA. MAGL inhibition may result in the activation of postsynaptic CB₂ receptors to decrease neuronal excitability and attenuate FCA via this mechanism. In line with this theory, a study by de Freitas and colleagues (2014) indicated a role for glutamatergic signalling in the PrL in mediating innate fear-induced antinociception. They found that administration of the NMDA receptor antagonist LY235959 directly into the PrL reduced innate fear-induced antinociception and panic-like behaviours elicited by GABA_A receptor blockade in the medial hypothalamus (de Freitas et al., 2014). Further studies are required to determine the mechanism of action of 2-AG/MAGL substrates in each of the three subregions of the mPFC in the regulation of FCA.

Region Administered	Drug Effects	
	FAAH Inhibition	MAGL Inhibition
IL	↓	↓
PrL	↑	↓
ACC	–	↓

Table 8.1: Summary of the effects of FAAH or MAGL inhibition in the IL, PrL and ACC on the expression of FCA in rats. – = no effect, ↓ = attenuated, ↑ = enhanced or prolonged. IL: infralimbic cortex; PrL: prelimbic cortex; ACC: anterior cingulate cortex

Region Administered	Drug Effects	
	FAAH Inhibition	MAGL Inhibition
IL	↓	–
PrL	–	–
ACC	–	–

Table 8.2: Summary of the effects of FAAH or MAGL inhibition in the IL, PrL and ACC on the expression of contextual freezing in rats. – = no effect, ↓ = attenuated, ↑ = enhanced or prolonged. IL: infralimbic cortex; PrL: prelimbic cortex; ACC: anterior cingulate cortex

The way in which individuals perceive pain can differ from person to person (Coghill et al., 2003). Therefore, understanding the influence of genetic background on pain is important for improved understanding and treatment of pain. In chapter 6, I studied the influence of genetic background on formalin-evoked nociceptive behaviour, contextual fear and FCA and determined if the behavioural effects seen were accompanied by alterations in the endocannabinoid system in the mPFC. Unlike SD rats, WKY rats exhibited reduced expression of FCA and greater formalin-evoked nociceptive behaviour. Moreover, both in the presence and absence of formalin-evoked nociceptive tone, WKY rats exhibited lower levels of contextually induced freezing and 22kHz ultrasonic vocalisation compared to SD rats. Formalin-treated WKY rats also had lower levels of AEA in the IL compared with SD rats. There was also a trend for increased levels of 2-AG in the IL and PrL of formalin-treated WKY rats compared to SD rats and a trend for increased levels of PEA and OEA in the IL of saline treated WKY rats compared to SD rats. These data are the first to suggest that WKY rats may have an impaired expression of FCA and conditioned fear-related behaviours compared to SD rats.

The form of fear-conditioning used in chapter 6 was contextual fear-conditioning. In contrast to the impaired expression of freezing behaviours exhibited in this paradigm, WKY rats have been shown to exhibit comparable freezing behaviour to both SD and other control strains following cued fear-conditioning (Ledoux et al., 1983, DaSilva et al., 2011, Schaap et al., 2013). While the neurocircuitry governing these forms of fear-conditioning is similar; involving the prefrontal cortex, amygdala and hippocampus, there are differences in how they are processed within these regions (Izquierdo et al., 2016, Chaaya et al., 2018). In contextual fear-conditioning the CS (context) is encoded in the hippocampus while in cued conditioning the CS (tone) is usually encoded by the lateral amygdala (Orsini and Maren, 2012, Phillips and LeDoux, 1995, Izquierdo et al., 2016). It is possible that this context-related hippocampal encoding is impaired in WKY rats leading to an impairment in the expression of fear behaviours upon contextual fear-conditioning. Moreover, the mPFC is involved in both forms of conditioning. In cued conditioning, the mPFC plays an important role in the retrieval of the aversive memory (Arruda-Carvalho and Clem, 2015b, Burgos-Robles et al., 2009, Corcoran and Quirk, 2007, Do Monte et al., 2016, Senn et al., 2014). In contextual fear-conditioning, upon damage to the hippocampus, prefrontal circuits takeover hippocampal function. This is known as the alternate pathway for contextual fear (Fanselow, 2010, Chaaya et al., 2018). This alternate pathway is key to forming contextual fear memories in the absence of hippocampal

control. It is possible that an impairment of hippocampal and mPFC circuitry leads to impaired expression of contextual fear in WKY rats. Further studies looking at these three fear-related brain regions (hippocampus; amygdala and PFC) may elucidate the mechanisms underlying the impaired expression of contextual fear in WKY rats. Optogenetic manipulation by stimulating or inhibiting the circuits within these regions in WKY rats and comparing the results to SD rats while measuring the levels of endocannabinoids and NAEs in these regions may be a promising strategy in this area.

The hyperalgesia exhibited by NFC WKY rats is in line with the previous work within our own group and among others showing that the WKY rats are hypersensitive to noxious formalin, thermal and mechanical stimuli (Burke et al., 2010, Taylor et al., 2001, O'Mahony et al., 2010, Gunter et al., 2000, Rea et al., 2014b, Madasu et al., 2016). FC WKY rats also exhibited robust hyperalgesia in the formalin test and impaired expression of FCA compared to SD rats. As FCA relies upon activation of the descending inhibitory pain pathway this result suggests that there may be reduced fear-induced engagement of the descending inhibitory pain pathway in WKY rats versus SD rats. In line with this work from our own group has shown a differential expression of *c-Fos* in the RVM and the DHSC in SD and WKY rats. Intra-IPAG administration of ACEA decreased formalin-evoked nociceptive behaviour in SD but not WKY rats, an effect that was associated with decreased *c-Fos* mRNA expression in the DHSC and increased *c-Fos* expression in the RVM in SD but not WKY rats, implying a dysfunction of the top-down control system in WKY rats (Jennings, 2015a). Regions within the descending inhibitory pain pathway, namely the mPFC, amygdala and PAG, are also involved in the expression of fear-related behaviour. It is possible that the circuitry or certain neuromodulators (endocannabinoids, GABA, glutamate) within these regions is different to SD rats leading to the hyperalgesia associated with WKY rats. Further studies targeting these regions are necessary to understand the neural mechanisms leading to hyperalgesia and impaired expression of FCA in the WKY rat strain.

In line with this thesis, I measured the levels of endocannabinoids and NAEs in the mPFC of formalin-treated FC and NFC rats. The results showed a significant strain effect for AEA in the IL with trends for lower levels of AEA in WKY rats, in particular NFC WKY rats. I hypothesised that the lower levels of AEA in the IL in the WKY rat may account for its hyperalgesic phenotype. This hypothesis was tested in chapter 7. In SD rats, increasing the endogenous tone of FAAH substrates including AEA via intra-IL administration of URB597, or injecting the stable AEA analog mAEA into the IL, reduced formalin-evoked nociceptive

behaviour, effects not observed in WKY rats. Intra-IL administration of the CB₁ receptor antagonist AM251 or the CB₁ receptor agonist ACEA had no effect on formalin-evoked nociceptive behaviour in either SD or WKY rats. As direct modulation of CB₁ receptors had no effect on the expression of formalin-evoked nociceptive behaviour it is possible that mAEA and FAAH substrates (via URB597) are acting at a receptor other than CB₁ to exert their effects in SD rats. If they were acting via CB₁ we might expect ACEA, the CB₁ agonist, to exert similar effects. As mAEA and URB597 did not exert any effect in WKY rats these results imply that there is a difference in the endocannabinoid system in the IL of WKY rats compared to SD controls. These differences could be at the level of baseline endocannabinoid tone, FAAH expression or a receptor-related function / mechanism. mAEA and URB597 could be exerting their effects via non-CB₁ related targets including CB₂ (Griffin et al., 2000b, Felder et al., 1996, Petrosino and Di Marzo, 2017), TRPV1 (De Petrocellis et al., 2001a, Di Marzo et al., 2001, Ross et al., 2001, Smart et al., 2000), PPARs (LoVerme et al., 2005, O'Sullivan and Kendall, 2010, Pistis and O'Sullivan, 2017, Okine et al., 2014) and GPR55 (Pertwee, 2007, Sharir and Abood, 2010, Ryberg et al., 2007, Kramar et al., 2017) and signalling through one or more of these targets in the IL may be different between the two strains. Further studies investigating the effects of mAEA and URB597 along selective agonists and antagonists for the above receptors may shed some light on this. Performing western blotting analysis to determine if receptor expression levels differ between these two strains would also be helpful.

While the work presented in this thesis has added to the body of knowledge surrounding the role of the endocannabinoid system in the emotional modulation of pain, there are certain limitations of the studies described herein which should be acknowledged.

- The levels of endocannabinoids and NAEs reported in this thesis were measured at a single time point and therefore it cannot be ruled out that alterations may have occurred at time points other than those examined in the studies. For example, we cannot conclusively rule out the possibility that MJN110 did not increase the levels of 2-AG in the IL and PrL at 45 minutes post injection. Moreover, mass spectrometry only measures the tissue levels of the analytes and not the released, extracellular signalling pool which may well have been altered by drug treatment. Studies employing *in vivo* microdialysis should be carried out to further investigate this possibility, although this methodology has proved challenging for researchers in the context of endocannabinoids given their high lipophilicity.

- The studies described in this thesis used only single doses of drugs. In particular the results in chapter 7 use only a single dose of mAEA, URB597, AM251 and ACEA. We cannot rule out the possibility that ACEA and AM251 may have influenced formalin-evoked nociceptive behaviour at a different dose. A dose response component in this experiment may have been informative but it was difficult to justify given that the doses were based on previous experiments where a drug effect was seen and desire to adhere to the principles of the 3Rs.
- I am aware that mRNA levels are not always translated or indicative of protein levels. mRNA and not protein levels were measured primarily due to methodological constraints including tissue amounts and the quality of antibodies available.

The work presented in this thesis provides a firm foundation for future studies aimed at further elucidation of the role of the endocannabinoid system in the mPFC in the emotional modulation of pain. Some additional suggestions for future work include:

- Determine the expression of CB₂ receptor protein in the mPFC using western immunoblotting, and the effects of FC thereon. Currently such an experiment is out of reach due to the lack of high quality antibodies for CB₂.
- Conclusively determine whether CB₂ is localised on GABAergic or glutamatergic neurons in the specific subregions of the mPFC to further our understanding of the neuronal mechanisms underlying CB₂ receptor-related modulation of pain- and fear-related behaviour.
- Determine the receptors by which FAAH and MAGL substrates in the IL and PrL exert their effects on pain, fear and FCA.
- Investigate the role of 2-AG and its receptors in the amygdala, PAG and RVM in formalin-evoked nociceptive behaviour, contextual fear and FCA.
- Determine whether the hippocampus or amygdala play a role in the strain differences exhibited by SD and WKY rats in fear, formalin-evoked nociceptive behaviour and FCA.
- Determine if any behavioural differences between SD and WKY rats following hippocampus and amygdala manipulation are accompanied by alterations in the endocannabinoid system within these regions.

- Determine the receptor mechanisms through which mAEA and URB597 exert their antinociceptive effects in SD rats and compare these to the WKY rat strain.

In conclusion, the work presented in this thesis indicates that the endocannabinoid system in the mPFC plays a key role in the emotional modulation of pain. The results also suggest that reduced engagement of endogenous analgesic mechanisms (FCA) contributes to the hypersensitivity to inflammatory pain in a genetic model associated with negative affect (the WKY rat). Differences in endocannabinoid system within the IL of WKY rats versus SD counterparts may also contribute to their hyperalgesic phenotype. These data advance our understanding of the role of the endocannabinoid system in pain, fear and their interaction and provide a solid foundation for future studies. Furthermore, these studies may inform the development of endocannabinoid-based therapeutics for the treatment of pain, fear or comorbid pain- and fear-related disorders.

Appendix

Buffers and Solutions for Western Immunoblotting

4X separation buffer (for 100ml)

- 1.5M Tris: 18.2g
- 4ml of 10% Sodium dodecyl sulphate (SDS; Sigma-Aldrich, Ireland, 20% solution diluted to 10%)
- Fill to final volume minus SDS as SDS forms bubbles, then add SDS)
- pH 8.8 with HCL

4X Stacking gel buffer (for 100ml)

- 0.05 M Tris: 6g
- 0.4% SDS: 4ml of 10% SDS (Sigma-Aldrich, Ireland, 20% solution diluted to 10%):
pH 6.8 with HCL

10X Running buffer (for 1L)

- Glycine (Sigma-Aldrich, Ireland): 144g
- Trizma-Base (Sigma-Aldrich, Ireland): 30g 100ml of 10 % SDS
- Dissolve in 1L of distilled water

10 X Transfer buffer

- Glycine (Sigma-Aldrich, Ireland): 144g
- Trizma-Base (Sigma-Aldrich, Ireland): 30g
- Make up to 1L distilled water

Making 9% separation gel for 2 gels

- 30% acrylamide (Sigma, Ireland): 6.0ml
- 4x sep buffer: 5ml
- Distilled water : 9ml
- 10% ammonium persulphate (Sigma, Ireland): 200µl
- TEMED (Sigma-Aldrich, Ireland) (in fume hood) : 20µl

Stacking gel for 10 ml (2 gels)

- 30% acrylamide (Sigma, Ireland): 1ml
- 4x stacking buffer: 2.5ml
- Distilled water : 6.5ml
- 10% ammonium persulphate (Sigma-Aldrich, Ireland): 100µl
- TEMED (Sigma-Aldrich, Ireland) (in fume hood) : 10µl

10x Tris-buffered saline (TBS) (1L)

- 200mM Trizma-Base (Sigma-Aldrich, Ireland), Ireland): 24.23g
- 1.37M NaCl (Fisher, Ireland): 80.06g
- Distilled water: 800mL
- Ph to 7.6 with HCL
- Make up to 1L with distilled water

Blocking solution: 5% non-fat dry milk (Aptamil, UK) in 0.05% Tween-20 (Sigma, Ireland) PBS

Primary Antibody: polyclonal rabbit antibody to the CB1 receptor (C-term) (1:200, catalogue no. 10006590; Cayman Chemical, MI) and mouse monoclonal antibody to b-Actin (1:10,000, A5441; Sigma-Aldrich, Dublin, Ireland) diluted in 5% milk/0.1% Tween PBS.

Secondary antibody: IRDye conjugated goat anti-rabbit (k800; 1:10,000, catalogue no. 926-32211; LICOR Biosciences, Oxford, UK) and goat anti-mouse (k700; (1:10,000; catalogue no. 926-68020; LICOR Biosciences, Oxford, UK) in 1% milk/0.05% Tween-20 PBS.

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